AD_____

Award Number: DAMD17-98-1-8656

TITLE: Biological Basis for Chemoprevention of Ovarian Cancer

PRINCIPAL INVESTIGATOR: Andrew Berchuck, M.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

REPORT DATE: October 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2002	Final (1 Oct 98 - 30 Sep 02)

4. TITLE AND SUBTITLE
Biological Basis for Chemoprevention of Ovarian Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8656

6. AUTHOR(S)
Andrew Berchuck, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, North Carolina 27710

E-Mail: berch001@mc.duke.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*
Prevention may represent a feasible approach to decreasing ovarian cancer mortality. To achieve a better understanding of the etiology of ovarian cancer, which can then be translated into more effective prevention strategies, we have initiated a prospective, population-based, case-control study in North Carolina that considers genetic susceptibility, epidemiologic risk factors and acquired genetic alterations. Subjects are interviewed in their homes and 474 cases and 488 controls have been accrued thus far. Blood and cancer samples have been collected and molecular analyses of (eg. p53, HER-2/*neu*) and genetic polymorphisms (eg. progesterone receptor) have commenced. We also have initiated an ovarian cancer chemoprevention program focusing on the progesterone receptor. Progestins have a potent apoptotic effect on ovarian epithelial cells and we have shown that levonorgestrel dramatically decreases ovarian cancer incidence in a chicken chemoprevention trial. In addition, we have shown that progestin mediated apoptosis in the ovarian epithelium is mediated by transforming growth factor-beta. With a recently awarded second program project grant from the DOD Ovarian Cancer Research Program, we will continue to work towards an understanding of the molecular epidemiology of ovarian cancer and towards development of effective chemoprevention strategies that might decrease mortality from this disease.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Ovarian Cancer	43
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Ovarian cancer is the fourth leading cause of cancer deaths among women in the United States. There are three potential approaches to decreasing ovarian cancer mortality: screening and early detection, more effective treatment and prevention. All of these avenues should be explored, but we believe that prevention represents the most feasible approach. The rationale for prevention is derived from epidemiologic studies that have examined the relationship between reproductive history, hormone use and ovarian cancer. It has been convincingly demonstrated that reproductive events which reduce lifetime ovulatory cycles are protective. Although most women are unaware of this protective effect, those who use oral contraceptive pills for more than 5 years or have 3 children decrease their risk of ovarian cancer by greater than 50%. The biological mechanisms that underlie the association between ovulation and ovarian cancer are poorly understood, however.

Our multidisciplinary ovarian cancer research group has been actively involved in studies that seek to elucidate the etiology of ovarian cancer and to translate this knowledge into effective preventive strategies. Joint consideration of genetic susceptibility, reproductive/hormonal and other exposures, acquired alterations in oncogenes and tumor suppressor genes and protective mechanisms such as apoptosis is required to accomplish this goal. We have initiated a molecular epidemiologic study of ovarian cancer in North Carolina to address the complex etiology of ovarian cancer.

In addition, we are actively involved in development of chemopreventive strategies. We have performed a study in primates that suggests that the oral contraceptive has a potent apoptotic effect on the ovarian epithelium, mediated by the progestin component. In addition, in subsequent studies performed *in vitro*, we have induced apoptosis in epithelial cells treated with the progestin levonorgestrel. Progestin mediated apoptotic effects may be a major mechanism underlying the protection against ovarian cancer afforded by OCP use. This forms the basis for an investigation of the progestin class of drugs as chemopreventive agents for epithelial ovarian cancer. Studies to test the progestin levonorgestrel in an avian model of ovarian cancer have been undertaken. In addition, we are exploring the molecular pathways that mediate progestin induced apoptosis in the ovarian epithelium.

Over the past four years with support from the DOD Ovarian Cancer Research Program we have made considerable progress, as will be summarized in this report. We have received an additional four years of support from a Program Project Grant from the DOD that will enable us to continue the projects that have been initiated during this initial funding period.

Body

Projects 1 and 2: Molecular-epidemiology of ovarian cancer

With the support of the Department of Defense Ovarian Cancer Research Program we have initiated a molecular epidemiologic study of ovarian cancer to work towards the goal of a better understanding of the etiology of ovarian cancer. Drs. Andrew Berchuck

(Gynecologic Oncologist) and Joellen Schildkraut (Epidemiologist) are working together to lead this study. Our initial plan was to accrue frozen tumor tissue and blood from 500 epithelial ovarian cancer cases treated at Duke University, the University of North Carolina at Chapel Hill and East Carolina University. In addition, 500 age and race-matched control subjects were to be accrued and both cases and controls were to be interviewed by telephone regarding known risk factors for ovarian cancer. After funding to support this project was received from the Department of Defense with Dr Berchuck as PI, additional funding was received to support this project from the NCI with Dr Schildkraut as PI. The additional funding has allowed us to increase the scope of the study such that nurse interviewers are visiting the homes of all the cases and controls to administer the study questionnaire. Research subjects are now accrued from hospitals in a 48 county region of central and eastern North Carolina using a rapid case ascertainment mechanism established through the state tumor registry. Prior to initiating the study, we had to go through the process of IRB approval in each of the various hospitals involved. Treating physicians are contacted by mail to request permission to approach potential research subjects. A letter is sent inviting a woman to participate only if permission to contact is granted. Three nurse interviewers were trained and the research questionnaire was field tested on 20 women with ovarian cancer. Final revisions to the questionnaire were made before the study began to accrue actual research subjects.

Our study uses a rapid case ascertainment system developed with the NC Central Cancer Registry (CCR), in which hospitals in the study area report ovarian cancer cases to the CCR within one month of diagnosis (see newsletter in appendix). Recently, several hospitals waived the requirement that active consent of the patient be obtained before the contact information was provided to our study staff. This change has increased our rate of accrual into the North Carolina Ovarian Cancer study from these hospitals. To date, the hospitals involved in the study have reported 628 women with ovarian cancer of whom 70 are pending. We have interviewed 474 of the 558 eligible women (response rate of 85%). To date, 808 potential controls have been identified through random digit dialing (RDD) screening, of which 749 have been contacted, and 59 are still pending. Of the 749 potential controls, 691 meet the eligibility requirements for the study. We have interviewed 488 controls in the study for a response rate of 71% (488/691). Demographic data on the first 337 cases and 426 controls is listed below in Table 1.

Table 1. NCOC Study Subjects

	Cases (n=337) n (%)	Controls (n=426) n (%)
Age		
20-49 years	140 (33)	174 (35)
50-74 years	286 (67)	382 (65)
Race		
Caucasian	362 (85)	425 (86)
African-American	52 (12)	60 (12)
Other	12 (3)	11 (2)

The investigators have had project meetings every month with all the research staff to review progress and address ongoing issues and at this point we are pleased with the accrual rate and other procedural aspects of the study. We continue to obtain blood specimens on over 99% of our study subjects. Of 456 paraffin block requests, 308 have been received, 118 are pending, and 30 are not available. A total of 97 frozen ovarian tumor samples have been received thus far. All clinical, epidemiologic and molecular data are stored as they are obtained in a computerized database. The pathologic features of the first 337 cases are listed below in table 2.

Table 2. Ovarian Tumor Characteristics (n=377)

	n (%)
Tumor Behavior	
Borderline	92 (22)
Invasive	332 (78)
Tumor Grade*	
I/II	114 (49)
III/IV	119 (51)
Tumor Stage*	
I/II	173 (42)
III/IV	238 (58)
Tumor Histology	
Serous	259 (61)
Endometrioid	47 (11)
Mucinous	53 (12)
Clear cell	21 (5)
Other	46 (11)

*2 missing tumor behavior, 15 stage, 193 tumor grade,

During the interview a thorough history of the menstrual cycle and reproductive experiences of the study participants is obtained assisted by the use a life-time calendar method. In addition, information on oral contraceptives and hormone replacement therapy is obtained. Data on the family history of cancer, other risk factors, and potential confounders is also collected. The interview takes 60-90 minutes to complete. The interactions between the nurses and subjects has been uniformly positive. The women with ovarian cancer are highly motivated to talk about their history and have a high level of interest in supporting a study aimed at increasing our understanding of the causes of ovarian cancer. They greatly appreciate the opportunity to talk with a nurse who is truly interested in hearing all the details of their life experience.

Previously, using ovarian cancer cases and controls from the CASH study, we found a strong association between high lifetime ovulatory exposure and alteration of the p53 tumor suppressor gene. In project 1 of this proposal, directed by Dr. Berchuck (Gynecologic Oncologist), we are seeking to confirm the association between high lifetime ovulatory exposure and alterations in p53. More broadly, we will attempt to

demonstrate that alterations in specific genes (eg, p53, HER-2/*neu*, c-*myc*) serve as molecular signatures of distinct etiologic pathways and allow definition of more homogenous subsets of ovarian cancer. This could be critical as we strive to develop prevention strategies, as the optimal means of prevention may vary between different subsets of these cancers. Ovarian cancer tissues have been collected and molecular analyses of the p53 tumor suppressor gene and HER-2/*neu* and c-*myc* oncogenes have commenced. In cases in which fresh frozen ovarian cancer tissue is not available, consent has been obtained to procure paraffin blocks. In the coming year we will merge the molecular and epidemiologic data from the first three hundred cases. This will allow us to address the goals of the specific aims outlined in this project involving definition of distinct subsets of ovarian cancer through their underlying molecular signatures.

P53 and HER-2/neu.

Immunhistochemical staining for p53 and HER-2/neu overexpression is ongoing. To date, 257 epithelial ovarian cancer cases have been immunohistochemically stained for p53 and 188 for HER-2/neu. Overexpression of p53 was seen in 33% of cases, including 41% of invasive cases and 8% of borderline tumors. Among invasive cancer cases, overexpression of p53 was strongly associated with advanced stage as our group and others have noted previously. Overexpression of p53 was seen in only 18% of stage I/II cases compared to 53% of stage III/IV cases.Only 8 of 188 cases (4%) were found to overexpress HER-2/neu. When limiting the analysis to invasive tumors, 5% were found to overexpress HER-2/neu. The frequency of HER-2/neu overexpression observed in this study is significantly less than was reported initially by some investigators, but there is a wide variance between studies, much of which may relate to differences in sensitivity and specificity of the methods employed. More recently with the development of anti-HER-2/neu antibody therapy (Herceptin) for breast cancers that overexpress this oncogene product, a standardized approach to immunohistochemical analysis of HER-2/neu overexpression has been accepted. The monoclonal antibody and immunostaining technique employed in this study were chosen specifically because they have become the accepted standard in testing breast cancers for overexpression of HER-2/neu. Using this technique, only cancers with HER-2/neu overexpression (greater than 2-3 fold) exhibit significant cell membrane staining and are scored as positive. Our finding that the fraction of ovarian cancers exhibiting overexpression was lower than expected is similar to that of a recent large study performed by the Gynecologic Oncology Group. In this study, 837 patients with recurrent disease were screened for overexpression to determine whether treatment with Herceptin would be appropriate, and only 11% of recurrent invasive epithelial ovarian cancers were found to have HER-2/neu overexpression. Although the frequency of overexpression in invasive cases was even lower (5%) in our study, this is not surprising given that HER-2/neu overexpression is associated with poor prognosis. Thus, a lower frequency of overexpression would be expected in our study, which includes all newly diagnosed cases, compared to the GOG study of patients with recurrent advanced ovarian cancer.

To date, we have characterized the p53 coding sequence on a total of 43 tumorsr. Of the 43 tumors, 14 were negative for a p53 immunostaining. Among these 14, 2 were found

to have a p53 mutation through sequencing. The following mutations were identified through sequencing: A276G, C135W, C176F, C242Y, D281V, del 140-41(T,C), F134L, H179R, insA 253, K132N, K132R, L194F, P278A, Q192, R175H, R213, R213R, R248Q, R273H, V157G, V173M, Y220C.

In project 2, initially under the direction of Dr. Futreal (Molecular Geneticist), we are examining the role of genetic susceptibility in the development of ovarian cancer. More recently, Dr. Futreal has left Duke and this project is now being led by Jeffrey Marks, Ph.D. (Molecular Biologist). Drs. Berchuck and Marks are co-directors of the Duke Comprehensive Cancer Center Breast/Ovarian Cancer Program and have a long track record of scientific collaboration over the past 10 years. Although most of the genes responsible for dominant hereditary ovarian cancer syndromes (eg. BRCA1/2) likely have been discovered, there is evidence to suggest that polymorphisms in other genes may also affect cancer susceptibility in a more weakly penetrant fashion. Drs. Marks and Berchuck will investigate whether genetic polymorphisms affect ovarian cancer susceptibility. These studies will focus on genes involved in pathways implicated in the development of ovarian cancer - such as hormone receptors. Since the effect of cancer susceptibility genes may be modified by other genes and exposures, he also will determine whether gene-gene and gene-environment interactions affect ovarian cancer susceptibility. Because of the low incidence of ovarian cancer, the ability to identify "high risk" subsets of women is critical if we hope to translate our emerging understanding of the etiology of ovarian cancer into effective prevention strategies.

It has been postulated that decreased activity of the progesterone receptor and vitamin D receptor or increased activity of the androgen receptor might increase OC risk. In view of this, we performed analyses of genetic polymorphisms in 301 cases (75% invasive, 25% borderline) and 358 controls in the North Carolina Ovarian Cancer Study (see table below). PCR-based methods were used to examine allele frequencies of polymorphisms in the progesterone receptor (PROGINS), vitamin D receptor (exon 9 Taq1 RFLP) and androgen receptor (exon 1 CAG repeat). Odds ratios (ORs) were computed and associations between genotypes and case/control status were assessed using logistic regression adjusting for age and race. There were no differences between cases and controls in mean age (54.0 years vs. 54.7 years) or fraction of African Americans (11% vs 14%). For the progesterone receptor polymorphism, ORs were 1.2 for heterozygotes (95% CI 0.8-1.7) and 0.8 for homozygotes (95% CI 0.4-1.8). For the vitamin D receptor polymorphism, ORs were 1.4 for heterozygotes (95% CI 1.0-2.0) and 1.1 for homozygotes (95% CI 0.7-1.8). The size of this study provides 80% power to detect ORs of 1.6 at a p=0.05 2-sided level. For the CAG polymorphism in the androgen receptor, there was no difference in mean allele lengths between cases (20.8 repeats) and controls (20.7 repeats). In addition, the frequency of either very long ($\geq$27) or a very short CAG alleles ($\leq$16) did not differ between cases and controls. None of the three polymorphisms were associated with age of OC onset or borderline vs. invasive histology. This study is not supportive of the hypothesis that polymorphisms in the progesterone receptor, vitamin D receptor or androgen receptor affect OC risk. We are also collecting epidemiologic data and in the future will examine whether nulliparity or other known risk factors are modified by these polymorphisms. The identification of

polymorphisms that increase OC risk is a worthwhile endeavor as this could facilitate identification of high-risk women who would be candidates for screening and/or prevention interventions designed to decrease mortality.

Table. ORs of allele types (blacks and whites only unless noted)

		Cases		Controls		Age and Race Adjusted	
Gene	Genotype	N	(%)	n	(%)	OR	95%CI
CYP3A4	Total	239	(100)	262	(100)		
	AA	192	(80)	218	(83)	1.0	Referent
	AB	29	(12)	31	(12)	1.1	(0.6 - 1.8)
	BB	18	(8)	13	(5)	1.5	(0.7 - 3.2)
CYP3A5	Total	239	(100)	262	(100)		
	AA	179	(75)	204	(78)	1.0	Referent
	AB	42	(18)	45	(17)	1.0	(0.6 - 1.6)
	BB	18	(8)	13	(5)	1.6	(0.7 - 3.3)
CYP2A6	Total	239	(100)	262	(100)		
	AA	203	(85)	222	(85)	1.0	Referent
	AB	35	(15)	38	(15)	1.0	(0.6 - 1.7)
	BB	1	(0)	2	(1)		
Progins	Total	292	(100)	369	(100)		
	11	202	(69)	265	(72)	1.0	Referent
	12	79	(27)	87	(24)	1.2	(0.8 - 1.7)
	22	11	(4)	17	(5)	0.8	(0.4 - 1.8)
Vitamin D	Total	295	(100)	372	(100)		
	TT	107	(36)	158	(42)	1.0	Referent
	Tt	145	(49)	152	(41)	1.5	(1.0 - 2.1)
	tt	43	(15)	62	(17)	1.0	(0.6 - 1.7)
MMP1	Total	328	(100)	393	(100)		
	G/G	94	(29)	102	(26)	1.0	Referent
	G/GG	154	(47)	207	(53)	0.8	(0.6-1.1)
	GG/GG	80	(24)	84	(21)	1.0	(0.7-1.6)
BRCA1-Q356R	Total	74	(100)	120	(100)		
	QQ	65	(88)	102	(85)	1.0	Referent
	QR	7	(9)	14	(12)	0.8	(0.3- 2.2)
	RR	2	(3)	4	(3)	0.7	(0.1- 4.4)
BRCA1-P871L	Total	318	(100)	395	(100)		
	PP	134	(42)	142	(36)	1.0	Referent
	LP	127	(40)	176	(45)	0.8	(0.5- 1.3)
	LL	57	(18)	77	(19)	0.8	(0.6- 1.1)
BRCA2 N372H	Total	316	(100)	390	(100)		
	AA:	175	(55)	223	(57)	1.0	Referent
	AC:	124	(39)	144	(37)	1.1	(0.8- 1.5)

	CC:	17	(5)	23	(6)	0.9	(0.5-	1.8)
Androgen Receptor		n=289		n=354				
	CAG1, mean (sd)	19.3	(2.7)	19.2	(2.1)			
	CAG1, mean (sd)	22.2	(2.8)	22.1	(2.7)			
	Average, mean (sd)	20.7	(2.5)	20.7	(2.1)			

Project 3: Chemoprevention

Project 3 is under the direction of Gustavo Rodriguez, M.D. (Gynecologic Oncologist). The prevention strategy outlined in our proposal is based on the observation that progestins have a potent apoptotic effect on ovarian epithelial cells. With regard to cancer prevention, the apoptosis pathway is one of the most important *in vivo* mechanisms that functions to eliminate cells that have sustained DNA damage and which are thus prone to malignant transformation. In addition, a number of well-known chemopreventive agents have been demonstrated to activate the apoptosis pathway in the target tissues that they protect from neoplastic transformation. We have performed a study in primates that suggests that the oral contraceptives (OCs) have a potent apoptotic effect on the ovarian epithelium, mediated by the progestin component. In addition, in subsequent studies performed in vitro, we have induced apoptosis in transformed, immortalized, cultured human ovarian epithelial cells treated with the progestin levonorgestrel. This suggests that progestins may have a direct apoptotic effect on the ovarian epithelium. The finding that progestins activate this critical pathway in the ovarian epithelium, the site where ovarian cancers arise, makes it likely that progestin mediated apoptotic effects are a major mechanism underlying the protection against ovarian cancer afforded by routine OC use. This forms the basis for an investigation of the progestin class of drugs as chemopreventive agents for epithelial ovarian cancer.

The studies outlined in our prevention grant are designed to add further support to notion that progestins are potent apoptotic agents on human ovarian epithelial cells, and to directly test the hypothesis in an animal model that progestins confer preventive effects against ovarian cancer. These aims in the grant are: (1) to evaluate the apoptotic effect of progestins on the human ovarian epithelium *in vivo*, (2) elucidate the molecular mechanisms by which progestins induce apoptosis in ovarian epithelial cells, and (3) to directly test the hypothesis that progestins confer preventive effects against ovarian cancer in a chemoprevention trial in the chicken, the only animal species with a high incidence of ovarian cancer.

Progress to Date:

Progestin Induces Apoptosis in the Ovarian Epithelium in Primates: There has been widespread belief that the ovarian cancer protective effect of OCP use is due to the ability of these agents to inhibit ovulation. We challenged this presumption because routine oral contraceptive use results in a disproportionately greater protective effect than that which can be solely attributed to ovulation inhibition. We hypothesized that the marked protective effect conferred by OCP's might be due to a potent biologic effect of contraceptive hormones on the ovary. To test this hypothesis, we performed a study in primates (cynomolgus macaques) designed to evaluate the long-term biologic effect of the contraceptive "Triphasil" on the ovaries (see ref's. 51, 116; appended manuscripts).

The remarkable similarity of the cynomolgus macaque to humans, particularly in regard to its 28-day menstrual cycle, makes this primate model ideal for designing experiments pertinent to human ovarian and reproductive biology. The purpose of our study was to search in the ovaries of contraceptive-treated monkeys for molecular changes that had the potential to be responsible for the known chemopreventive effects of oral contraceptives. Given the importance of the apoptosis pathway *in vivo* for cancer prevention, we elected to investigate whether long-term oral contraceptive exposure induced apoptosis in the primate ovarian epithelium.

Eighty animals were prospectively randomized into four groups including a control group, a group treated with Triphasil (which contains the estrogen ethinyl estradiol and the progestin levonorgestrel), and one group each treated either with ethinyl estradiol or levonorgestrel alone on the same dosage and schedule as those animals receiving Triphasil. The animals were maintained on the monthly contraceptive hormone schedule for three years. During the third week of the last month of the study, the animals were sacrificed; the ovaries were removed, formalin-fixed, sectioned, and then examined for morphologic and immunohistochemical evidence of apoptosis by observers blinded to treatment group. For each ovarian section, the percentage of epithelial cells undergoing apoptosis was quantified. The results are summarized in Table 1 below. As compared to control and ethinyl estradiol-treated monkeys, a striking and statistically significant increase in apoptosis was noted in the ovarian epithelium of monkeys treated with Triphasil ($p<.01$) or levonorgestrel ($p<.001$), with the maximal effect (six-fold) seen in the group treated with levonorgestrel alone. The degree of apoptosis was not different between ethinyl estradiol-treated monkeys and controls. These data demonstrate the novel finding that oral contraceptive exposure markedly induces apoptosis in the ovarian epithelium, and that the progestin component of the pill is responsible for this effect. This discovery formed the basis for the studies proposed in this grant, designed to investigate progestins as potential ovarian cancer chemopreventive agents.

Table 1.

Apoptotic Effect of Hormone Treatment on Macaque Ovarian Epithelium

Study Group	Number of Animals/Group	Median Percent of Apoptotic Epithelial Cells	Range of % of Apoptotic ells
Control	20	3.9%	0.1 - 33.0%
Hormone Treated			
Ethinyl-Estradiol	20	1.8%	0.1 - 28.6%
Combination Pill	17	14.5%	3.0 - 61.0%
Levonorgestrel	18	24.9%	3.5 - 61.8%

Multiple Comparisons:
Control - Levonorgestrel ($p<0.001$)
Combination Pill - Ethinyl-estradiol ($p<0.001$)
Ethinyl-Estradiol - Levonorgestrel ($p<0.001$)
Control-Combination Pill ($p=0.01$)

Progestin Induction of Apoptosis in the Ovarian Epithelium in Primates is Associated with Differential Regulation of Transforming Growth Factor-Beta:

The discovery that progestin markedly induces apoptosis in the ovarian epithelium led us to search for factors that regulate apoptosis in the ovarian epithelium. TGF-β has been implicated in the apoptotic pathway of a variety of cell types including hormonally sensitive epithelia such as the breast and prostate. In addition, well-known cancer preventive agents such as the retinoids and the anti-estrogen Tamoxifen have been shown to induce TGF-β expression in the target tissues that they protect, including epithelial cells in the upper aero digestive tract and breast. Interestingly, multiple members of the steroid superfamily including the retinoids, vitamin D, and estrogens have been shown to modulate expression of TGF-β and the promoter region for specific TGFβ isotypes contains features suggesting hormonal control.

Given the known importance of TGF-β as a regulator of apoptosis and as a potential mediator of action of other chemopreventives, we decided to examine whether progestins regulate TGF-β expression in the ovaries of primates from the trial described above. Primate ovarian sections from the four treatment groups noted above were stained immunohistochemically with monoclonal antibodies reactive with either TGF-β1 or TGF-β2 and TGF-β3 (TGF-β2/3). The ovarian sections were examined by two independent sets of reviewers, all of who were blinded to the hormone administration data. Staining for TGF-β was evaluated in 4 separate ovarian compartments of each study slide (ovarian surface epithelium, primordial oocyte cytoplasm, granulosa cells of tertiary follicles, and endothelium in ovarian hilar vessels) and graded according to degree of staining intensity from 0-3+ (TGF-β1) and 0-4+ (TGF-β2/3). High expression of TGF-β1 was defined by the slide reviewers as 2+ to 3+ staining intensity, whereas high expression of TGF-β2/3 was defined as 3+ to 4+ staining intensity. Three ovarian sections in the TGF-β1 staining group, and two ovarian sections in the TGF-β2/3 staining group were excluded from grading because the samples were technically insufficient for evaluation.

The quantitative results are summarized in Tables 2 and 3. Progestin treatment, either combined with estrogen (Triphasil group) or administered alone (levonorgestrel group) was associated with a striking and highly statistically significant decrease in expression of TGF-β1 in the ovarian epithelium ($p< 0.001$), and a moderate decrease in expression of TGF-β1 in the oocyte cytoplasm ($p= 0.002$). (Table 2.) In contrast, progestin treatment was associated with a marked increase in expression of TGF-β2/3 in the ovarian epithelium ($p< 0.001$). Without exception, TGF-β2/3 expression in the ovarian epithelium was high (3-4+ staining) in every monkey on progestin (N=34). Similarly, there was a significant increase in TGF-β2/3 expression in the ovarian hilar endothelial cells in monkeys on progestin. ($p< 0.001$) In contrast, progestin treatment was associated with a marked decrease in TGF-β2/3 expression in granulosa cells ($p < .001$). (Table 3.)

Table 2.
Hormone Regulation of TGF-β_1 Expression in the Macaque Ovary

Numbers (%) ovaries/treatment group with high TGF-β_1 expression (2-3+) in each ovarian compartment

	Epithelium	Granulosa Cells	Ooctyes	Endothelium
Treatment Group				
Control	18 (90%)	7 (35%)	7 (35%)	0 (0%)
Ethinyl Estradiol	16 (84%)	4 (21%)	2 (13%)	0 (0%)
Triphasil	3 (19%)	2 (13%)	0 (0%)	0 (0%)
Levonorgestrel	1 (6%)	2 (12%)	0 (0%)	0 (0%)
Overall approximate exact test:	p<.001	.31	.002	1.00
Triphasil/Levonorgestrel versus Control/ Ethinyl Estradiol	p<.001	.15	.003	1.00

Table 3.
Hormone Regulation of TGF-β2/3 Expression in the Macaque Ovary

Numbers (%) ovaries/treatment group with high TGF-β expression (3-4+) in each ovarian compartment

	Epithelium	Granulosa Cells	Ooctyes	Endothelium
Treatment Group				
Control	6 (32%)	12(63%)	14 (74%)	5(26%)
Ethinyl Estradiol	2 (10%)	8(38%)	17(81%)	3(23%)
Triphasil	17(100%)*	1(6%)*	16(94%)	16(94%)*
Levonorgestrel	17(100%)*	1(6%)*	14(82%)	16(94%)*

* P<.001, approximate exact test. Pair wise comparisons of Triphasil/Levonorgestrel versus Control/EE groups were statistically significant (p<.001) for all compartments except Oocytes, except for the Granulosa comparison with Ethinyl Estradiol (p=.03).

Table 4.

Relationship Between Treatment, TGF-β Expression and Apoptosis in the Macaque Ovarian Epithelium

Treatment	TGF-β_1 N	% 2-3+	Mean proportion of apoptotic cells in ovarian epithelium (SE)	TGF-$\beta_{2/3}$ N	% 3-4+	Proportion of apoptotic cells in ovarian epithelium (SE)
Control	20	90%	6.3 (1.6)	19	32%	6.4 (1.7)
Ethinyl Estradiol	19	84%	6.2 (2.1)	20	10%	4.5 (1.6)
Triphasil	16	19%	22.3 (4.1)**	17		100% 21.2(4.0) ***
Levonorgestrel	17	6%	25.1 (4.3)**	17	100%	26.4 (4.1)**

SE indicates standard error

** p < .001 by Dunnett's test of mean apoptotic index for treatment with Control

*** P = .002 by Dunnett's test of mean apoptotic index for treatment with Control

Within the ovarian epithelial compartment, comparison of the apoptotic index with the degree of change in the expression of the TGF-β isoforms revealed a significant correlation between changes in TGF-β expression and apoptosis (Table 4). The Pearson correlation between the proportion of high TGF-β expression and the mean proportion of apoptotic cells across treatments was -.998 (p=.002) for TGF-β1 and .973 (p=.03) for TGF-β2/3. Finally, overall, there was a negative association between TGF-β2/3 overexpression and TGF-β1 overexpression (kappa = -.62; p<.001). Taken together, these data demonstrate the novel finding that progestin-induced apoptosis in the ovarian epithelium is associated with an isotype switch in expression of TGF-β. These data were published this year (JNCI 2002; 94:50-60)

Progesterone receptor is expressed by the human ovarian epithelium:

The discovery that progestins induce apoptosis and differentially regulate TGF-β in the ovarian epithelium led us to search for potential mechanisms of action underlying these effects. It is possible that progestins induce the expression of factors in the ovarian stroma, which then induce apoptosis and impact TGF-β expression via a paracrine effect in the adjacent ovarian epithelium. Conversely, it is possible that progestins exert direct biologic effects on the ovarian epithelium, mediated by the progestin receptor. Prior to testing the hypothesis that progestins have a direct biologic effect on the ovarian epithelium, we examined the human ovarian epithelium for expression of the progestin receptor. Immunohistochemical staining for progesterone receptor was performed on normal ovarian tissue samples obtained from 40 women who underwent oophorectomy as

part of a gynecologic procedure performed for benign gynecologic indications. The progesterone receptor was found to be uniformly expressed by the ovarian epithelium in all cases, including the ovaries from both pre- and post-menopausal women. In addition, progesterone receptor expression was detected in the ovarian epithelium lining inclusion cysts trapped within the ovarian stroma. In separate experiments, we have demonstrated expression of both the A and B isoforms of the progestin receptor *in vitro* in several ovarian cancer cell lines (OVCA 420,429,432,433; OVCAR 3, DOV-13), as well in cell cultures derived from non-malignant human ovarian epithelium. Although the physiologic role of the progesterone receptor within the ovarian epithelium remains to be elucidated, localization of progesterone receptor to the ovarian epithelium suggests a functional role for progestins in ovarian epithelial cells.

Progestin Induces Apoptosis in Human Ovarian Epithelial Cells *in vitro*.

Having seen consistent expression of the progesterone receptor in the ovarian epithelium, we have tested the hypothesis that progestins have a direct apoptotic effect on the ovarian epithelium. We have tested the growth and apoptotic effects of a variety of progestins on cells derived from the human ovarian epithelium, including immortalized ovarian epithelial cultures as wells as ovarian cancer cell lines. We have been able to demonstrate potent inhibition of growth and induction of apoptosis with progestins. We are currently still working on elucidating the molecular signaling events underlying this inhibitory effect.

Progestin Treatment Decreases the Number of Tumors in Egg Laying Hens:

The discovery that progestin induces apoptosis in the ovarian epithelium led us to speculate that progestin-mediated biologic effects may underlie the protective effects of OCP's against ovarian cancer, rather than ovulation inhibition as had been previously suggested. This opened the door for consideration of the progestin class of drugs as candidate preventive agents for ovarian carcinoma. Furthermore, we speculated that a preventive approach using progestins might be possible in menopausal women, who by definition do not ovulate. To test this hypothesis, we created a menopausal chicken model and performed a two-year prevention trial to evaluate progestins as chemopreventives for ovarian cancer. Two thousand four hundred two year-old birds were randomized into six groups (400 each), with hormonal and dietary manipulation as follows:

1) Full-fed control,
2) Feed restricted control (feed restriction to maintain pullet weight, but below threshold required for ovulation,
3) Feed restricted, with diet enriched with Vitamin D,
4) Feed restricted plus the progestin levonorgestrel,
5) Feed restricted, plus levonorgestrel, with diet enriched with Vitamin D,
6) Feed restriction plus the progestin Provera.

Caloric restriction to a diet that maintains pullet weight induces an anovulatory state, and causes marked regression of the whole reproductive tract in the chicken. Therefor, Groups 2-6 were anovulatory.

The primary objective of the study was to evaluate progestins as ovarian cancer preventives. Outcome measures included time of onset and incidence of ovarian adenocarcinomas. A secondary objective was to evaluate whether a modest dietary enrichment with Vitamin D would confer ovarian cancer protection, or confer additional ovarian cancer protection to that provided by progestin. The doses of levonorgestrel and Provera were administered in amounts comparable to that in OCP and hormone replacement regimens in women, or approximately human equivalent doses of .125 mg/day levonorgestrel, and 5mg/day of Provera. Group 1 was given a regular diet, and therefor was expected to continue to ovulate throughout the trial.

The trial has been completed, and tumors accrued during the trial have undergone a meticulous pathologic review over the past 18 months by a team comprised of both avian and human gynecologic pathology and veterinary expertise. Results from the trial show a marked decrease (66%) in reproductive tract tumors (ovarian and oviductal) in anovulatory birds versus ovulatory birds (group 2 versus group1), from 33% in full fed birds (group 1) to approximately 11% in feed restricted birds (Group 2). The reduction in the incidence of reproductive tract tumors in each of the groups at the trial completion, relative to the appropriate feed-restricted control group (Group # 2), is as follows:

Treatment Group	**% Reduction in Reprod. Tract Tumors**
Group 3: Feed restricted, plus Vitamin D	14%
Group 4: Feed restricted plus levonorgestrel	36%
Group 5: Feed restricted, plus levonorgestrel, plus Vitamin D	42%
Group 6: Feed restriction plus Provera	32%.

More importantly, when looking at the impact of chemopreventive exposure on the outcome of ovarian cancer incidence, as defined by tumors that are clearly primary ovarian cancer, the reduction in ovarian cancer incidence by group, as compared to the appropriate feed restricted control group (Group #2), is shown below:

Treatment Group	**% Reduction in Primary Ovarian Cancers**
Group 3: Feed restricted, plus Vitamin D	25%
Group 4: Feed restricted plus levonorgestrel	41%
Group 5: Feed restricted, plus levonorgestrel, plus Vitamin D	72%
Group 6: Feed restriction plus Provera	62%.

Results suggest at least 50% fewer reproductive tract tumors in the progestin-treated groups (groups 4-6) versus control (group 2). Thus, the study suggests an ovarian-cancer-protective effect of progestins, unrelated to ovulation. Interestingly, the lowest tumor incidence occurred in the group of birds receiving progestin and a Vitamin D enriched diet. These data are supportive of the notion that a combination of candidate ovarian cancer preventives may confer enhanced ovarian cancer preventive effects as compared to use agents used singly.

Progestin-Potent OCs Enhance Protection against Ovarian Cancer in Women. We have recently completed a re-analysis of data from the Cancer and Steroid Hormone (CASH) study, a very large case-control study from the early 1980's that demonstrated that women who use OCs have a significant reduction in the risk of ovarian cancer. We hypothesized that if the protective effects of OCs are related to the progestin component, we would expect that progestin potent OCs may be more effective at preventing ovarian cancer than OC formulations containing weak progestins. To test this hypothesis, we reanalyzed data from the CASH study, specifically investigating the relationship between the progestin and estrogen potency in combination oral contraceptives that women took in the CASH study, and the risk of developing ovarian cancer. When comparing OCs categorized by estrogen and progestin potency in 400 ovarian cancer cases and 3000 controls, our results provide statistically significant evidence that OC formulations with increased progestin potency confer twice the reduction in risk of ovarian cancer than those with lower progestin potency, irrespective of the estrogen content ($p<0.001$). The analyses also demonstrated a significant reduction (60-70%) in risk of ovarian cancer associated with exposure to high progestin potency OCs even among women who used OCs for a relatively short duration (less than 18 months). The finding that the degree of protection afforded by OCs is related to progestin potency is consistent with the hypothesis that biologic effects related to the progestin component may be a key mechanism underlying the reduction in ovarian cancer risk associated with OC use. This study was published this year (JNCI 2002; 94: 32-8)

Synopsis of Data:

We have discovered that progestins markedly induce programmed cell death (apoptosis) and up-regulate expression of Transforming Growth Factor Beta (TGF-β) in the ovarian epithelium. These two molecular events have been strongly implicated in cancer prevention *in vivo*, and are believed to underlie the protective effects of other well-known chemopreventive agents such as the retinoids and Tamoxifen. The finding that progestins induce these two molecular events in the ovarian epithelium, the target site for ovarian cancer development, leads us to believe that progestin-mediated biologic effects may be a major mechanism underlying the marked protective effect of OCP's against ovarian cancer. This forms the basis for an investigation of progestins as chemopreventive agents for ovarian cancer.

We believe that our discovery may lead to a whole new indication for use of progestin compounds: namely, as chemopreventive agents for ovarian cancer. Our discovery opens the spectrum of use of these agents as preventives to all women, including the elderly, who have the highest age-specific incidence of ovarian cancer. Most importantly, if the protective effect of OCP's is due to a progestin-mediated biologic effect, rather than ovulation inhibition, then it should be possible to develop a highly effective pharmacologic strategy using progestins in women who are post-menopausal (who by definition do not ovulate) and who represent the group at greatest risk of developing ovarian cancer. It is interesting to speculate that if routine OCP use can reduce ovarian cancer risk by 50%, that a pharmacologic strategy that exploits the mechanism of action underlying the protective effects of OCP's could achieve even greater protective effects, leading to improved longevity and quality of life for women.

Our preliminary experience with the chicken supports the feasibility of this animal model for ovarian cancer research. Similar to humans, we have demonstrated a strong association between ovulation and ovarian cancer incidence in chickens (80% decrease in reproductive tumors, in Group 2 versus Group 1 in Trial above; Group 2 had 50% fewer lifetime ovulations than Group 1). This suggests a common pathogenesis for human and chicken ovarian cancers, related to ovulation-induced genetic damage to the ovarian epithelium. In addition, our first prevention trial has yielded preliminary evidence suggesting an ovarian cancer protective effect from our intervention with progestins, and a possible additive protective effect from Vitamin D.

As we go forward, we plan to continue to elucidate the molecular events associated with progestin action in the ovarian epithelium. We will also seek to gather further evidence to validate the chicken ovarian cancer animal model, and to plan to expand the focus our investigation to include other candidate chemopreventive that also activate cancer preventive changes in the ovarian epithelium. We speculate, that a chemopreventive formulation that includes two or more agents, working by similar or dissimilar pathways may have additive or synergistic preventive effects, thereby potentially leading to enhanced ovarian cancer prevention, while minimizing toxicity.

Key research accomplishments

We have gathered significant pre-clinical evidence in support of progestins as potential ovarian cancer preventive agents. Our research is now extending to the evaluation of other candidate agents for the prevention of ovarian cancer.

1) We have accrued a large number of cases and controls to a prospective, population-based, case-control study of ovarian cancer in North Carolina. Blood and tissue samples and epidemiologic data have been accrued as well. Analyses of genetic susceptibility polymorphisms and molecular epidemiologic signatures are ongoing.

2) We have discovered that progestins markedly activate TGF-β signaling pathways in the ovarian epithelium in primates, and that these effects are highly associated with apoptosis. We are now performing studies *in vitro* designed to characterize the complex biologic effects of progestins and other candidate preventive agents on apoptotic and TGF-β signaling pathways in ovarian epithelial cells, and seek to determine whether TGF-β mediates the apoptotic effect of progestins on the ovarian epithelium.

3) Our avian chemoprevention trial has been completed. An avian patholoist and gynecologic pathologist have been performing a meticulous evaluation of the tumors accrued during the trial. Our preliminary data suggests a 35-50% reduction in reproductive tract tumors in our progestin-treated chickens as compared to appropriate controls. In addition, we have some evidence that the combination of a Vitamin D-enriched diet and progestin treatment might have enhanced ovarian cancer preventive effects over progestin alone.

4) We have performed a reanalysis of data from the Cancer and Steroid Hormone Study (CASH), leading to the finding that progestin-potent oral contraceptives confer enhanced protection against ovarian cancer as compared to progestin-weak oral contraceptives. These are the first human data directly linking the progestins in oral contraceptives to an ovarian cancer protective effect.

Reportable outcomes

1) Polymorphisms in the progesterone, androgen and vitamin D receptors do not increase risk of ovarian cancer.

2) Progestin induction of apoptosis in the macaque ovarian epithelium is associated with differential regulation of transforming growth factor-β.

3) Combination OC formulations with high progestin potency may confer greater protection against ovarian cancer than those with low progestin potency.

Conclusions

The studies initiated by our program will enable us to define more homogeneous subsets of ovarian cancer based on epidemiologic and molecular characteristics, to identify women who are at increased risk for this disease and to develop chemopreventive strategies designed to decrease ovarian cancer incidence and mortality. We anticipate that much of our data will grow to maturity in the coming few years with continued support from the DOD Ovarian Cancer Research Program.

References

1) Rodriguez GC, Nagarsheth N, Rex C. Bentley, Walmer DK, Cline M, Whitaker RS, Eisner P, Berchuck A, Dodge R, Adams M, Hughes CL: Progestin Induction of Apoptosis in the Macaque Ovarian Epithelium is Associated with Differential Regulation of Transforming Growth Factor-Beta. J Natl Cancer Inst 2002;94:50-60.

2) Schildkraut J, Caligert B, Rodriguez GC. The Impact of Progestin and Estrogen Potency of Oral Contraceptives on Ovarian Cancer Risk. J Natl Cancer Inst 2002;94:32-8.

3) Rodriguez GC, Carver D, Anderson K, Barnes J, Berchuck A, Whitaker R, Eisner P, Petitte J. Evaluation of Ovarian Cancer Preventive Agents in the Chicken. (Abstract) 2001 Annual meeting of the Society of Gynecologic Oncologists (best poster award).

4) Berchuck A, Lancaster J, Calingaert B, Wenham R, McLean K, Halibi S, Marks J, Schildkraut J. A case-control study of candidate ovarian cancer susceptibility polymorphisms. (Abstract) 2002 Annual meeting of the Society of Gynecologic Oncologists (accepted for oral presentation).

5) Lancaster, JM, Wenham R, Halabi S, Calingaert B, Marks JR, Moorman PG, Bentley RC, Berchuck A, Schildkraut JM. Relationship between ovarian cancer risk and progesterone receptor gene polymorphism (PROGINS) in population-based, case-control study in North Carolina. (under review)

Appendices

September 2002 —Vol. 4, No. 1

North Carolina Ovarian Cancer Study

PURPOSE OF STUDY:

To identify the environmental, reproductive, and genetic factors that contribute to the development of ovarian cancer.

STUDY PERIOD: 1999-2003

STUDY ELIGIBILITY CRITERIA:

Diagnosis of primary epithelial ovarian cancer (including borderline) or primary peritoneal cancer;

Patient must be between the ages of 20 and 74 at diagnosis;

Patient must reside within 48-county study region (see map on p.3).

Also In This Issue...

September is Gynecologic Cancer Awareness Month

Rapid-Case Ascertainment and the NCOCS

The *North Carolina Ovarian Cancer Study (NCOCS)* is one of the largest population-based, epidemiologic, case-control studies on ovarian cancer in the nation. Funded by the National Institutes for Health (NIH) and the Department of Defense, study enrollment began in 1999 and will continue through at least June of 2003.

One of the most crucial elements to a study of this caliber is a sound system for rapid-case ascertainment. In a recent letter to hospital cancer registrars, Dale Herman, Director of the North Carolina Central Cancer Registry, outlined how the rapid-case ascertainment helps to both ensure valid data analysis and avoid research biases. In his letter, Dr. Herman wrote that, for rapid-case ascertainment purposes:

"We request hospitals, labs, or physicians to report new diagnoses to the CCR in abbreviated form within a 'rapid' reporting cycle (i.e., bi-weekly or monthly, in addition to the regular quarterly reports.) These rapid case ascertainment reports typically consist of simply a copy of the pathology report(s), case identifying information, and the name of the physician. The reporting format is intentionally flexible, and CCR staff from the RCA core work directly with individual institutions to identify the easiest approach for that institution, including electronic reporting."

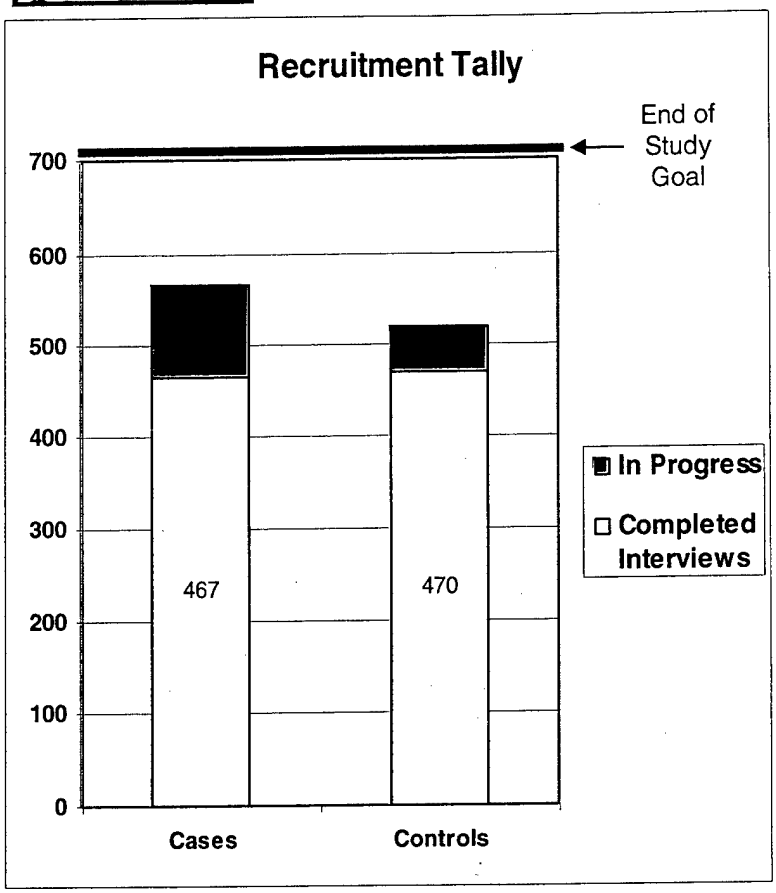
Rapid-case ascertainment facilitates the identification of women with newly diagnosed ovarian cancer within just a few months of their diagnosis. Hospital cancer registrars are asked to report all newly diagnosed **primary epithelial ovarian cancer (*including* borderline)** and **primary peritoneal cancer** cases between the ages of 20 and 74 to the North Carolina Central Cancer Registry on the bi-weekly or monthly basis described above. This process allows the *NCOCS* study team to contact newly diagnosed women as soon as possible, instead of waiting as long as six months. The NCOCS study team appreciates the effort the CCR has put into facilitating our research. We would also like to thank all the hospitals in our study region that have arranged a system of RCA reporting.

In Memoriam

Marilyn F. Vine (August 2, 1956-September 4, 2002)

We would like to dedicate this issue to our friend and colleague, Marilyn Vine, who recently lost her battle with ovarian cancer. Marilyn was an epidemiologist at Duke University whose primary research interest was in the area of early detection of ovarian cancer. We will miss her.

NCOCS Accrual Report September, 2002



Study Accrual Remains *High*

Women with ovarian cancer continue to participate in the *North Carolina Ovarian Cancer Study* at impressively high rates. Our remarkable response rate of 85% is due largely to the dedication of all the Gynecologic Oncologists and Cancer Registrars in our study area who have been participating in the rapid-case ascertainment of newly diagnosed ovarian cancer patients. The study team appreciates the efforts of both the Cancer Registrars and the rapid-case ascertainment team at the Central Cancer Registry!

Since April 1999, 32 hospitals across our 48-county study region have sent the North Carolina Central Cancer Registry the names of 662 women recently diagnosed with ovarian cancer. From January to August of this year, we have already received 165 names, compared to 167 in all of 2001. While the response rate among these women is very encouraging, we are still falling short of our overall goal of completing interviews with 700 affected women by the study's end. We will need the continued help of all participating hospitals in our study region if we are to reach this goal. If you have any questions or ideas, or if you are in our 48-county region and would like your hospital to participate in our study, please contact Christine Lankevich at 1-888-246-1250.

Thank You!!

We would like to thank the following hospitals for sending cases to the North Carolina Ovarian Cancer Study:

Alamance Regional Hospital
Betsy Johnson Memorial Hospital
Cape Fear Valley Health System
Carolinas Medical Center
Catawba Memorial Hospital
Central Carolinas Medical Center
Chatham Hospital
Craven Medical Center
Duke University Medical Center
Durham Regional Hospital
ECU (Pitt County Memorial Hospital)
FirstHealth Moore Regional Center
Forsyth Medical Center—Novant Health
Heritage Hospital
High Point Regional Hospital
Iredell Memorial Hospital
Johnston Memorial Hospital
Lincoln Regional Medical Center
Lenoir Memorial Hospital
Maria Parham Hospital
Moses H. Cone Memorial Hospital
Nash Health Care
New Hanover Regional Med. Ctr.
Northeast Medical Center
Presbyterian Hospitals
Rowan Regional Medical Center
Rex HealthCare
Sampson Regional Medical Center
Stanly Memorial Hospital
UNC Hospitals
Wake Medical Center
Wilson Memorial Hospital

Ovarian Cancer
By Marilyn Vine, Ph.D.

Listen to me; I'm cancer free.
Or so I thought the truth to be.
To my surprise, a lump grapefruit size
had grown on my ovary.

Signs were there, but not so rare.
Little did I know that I should care.
Bloating and gas, common symptoms that pass,
lasted longer than I could bear.

My doctor was wise. Before I did rise,
he performed a pelvic exam.
Then on ultrasound, a tumor was found.
It wasn't my gallbladder. Damn!

Surgery, chemotherapy,
treatments of choice the experts agree.
Not too much fun, but the battle is won,
at least temporarily.

No screening test is considered the best.
So, be aware of signs that may manifest.
If symptoms last, see a doctor real fast
and pray for a better lab test.

September is Gynecologic Cancer Awareness Month
Resources on the Web:

Gynecological Cancer Foundation:
www.wcn.org/gcf

National Ovarian Cancer Coalition:
www.ovarian.org

Women's Cancer Network:
www.wcn.org

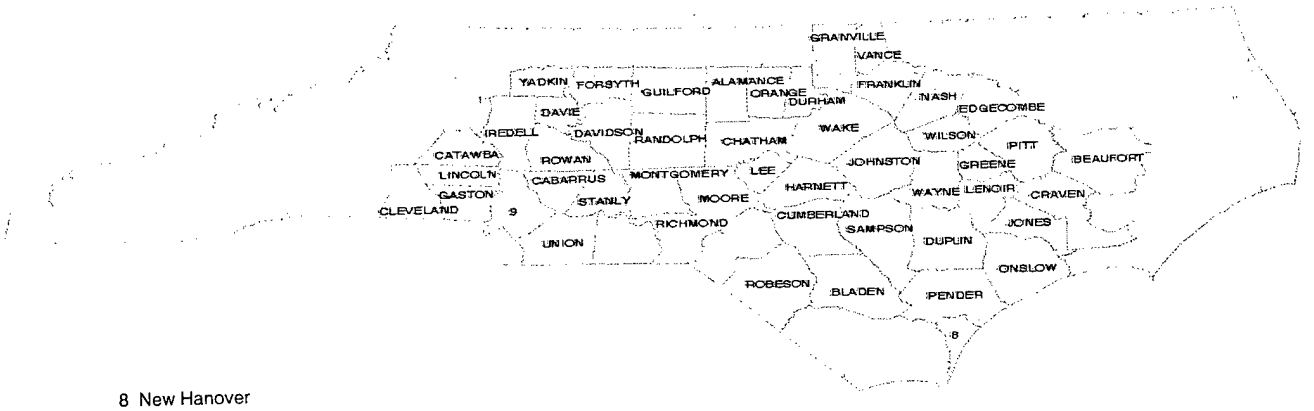
OncoLink: Ovarian Cancer:
www.oncolink.upenn.edu

Ovarian Cancer National Alliance:
www.ovariancancer.org

National Ovarian Cancer Coalition Triangle Chapter (TriNOCC):
www.trinocc.org

48-County Study Area

If you are in the study area and would like your hospital to participate call Christine Lankevich, 1-888-246-1250



8 New Hanover
9 Mecklenburg

STUDY CONTACT INFORMATION

DUKE COMPREHENSIVE CANCER CENTER

CANCER PREVENTION, DETECTION, AND CONTROL RESEARCH PROGRAM

[redacted]

- **Andrew Berchuck, M.D.**
  Professor, Division of Gynecologic Oncology
  (919) 684-3765
  email: berch001@mc.duke.edu

- **Joellen Schildkraut, Ph.D.**
  Associate Professor
  (919) 681-4761
  email: schil001@mc.duke.edu
- **Christine Lankevich, MPH**
  Project Manager
  1-888-246-1250
  (919) 681-4554
  email: lanke001@mc.duke.edu

- **Dale Herman, Director**
  (919) 715-4555
  email: dale.herman@ncmail.net
- **Dianne Vann, Research Associate**
  (919) 715-4560
  email: dianne.vann@ncmail.net
- **Gloria Regan, Research Associate**
  (919) 715-4562
  email: gloria.regan@ncmail.net

Related Ovarian Cancer Research

Duke is participating in a nation-wide pilot study called the **"Ovarian Cancer Screening Pilot Trial in High Risk Women"**. The goal of this study is to recruit 2,500 women to find out whether CA125 - a chemical in a woman's body - is helpful in finding early ovarian cancer among women at higher risk for this disease. This study measures women's CA125 levels every three months over one to two years.

Being in the study includes:

- signing consent and medical record release forms,
- having your blood drawn every three months for 1-2 years,
- completing a study survey at each visit, and
- some women are asked to have an ultrasound exam.

Women *may* be eligible for this study if they are over 30 years old, have a strong family history of breast or ovarian cancer, have an inherited alteration in genes known as BRCA1 and BRCA2, or have a close relative with such a mutation.

For more information or to refer a patient to this study, contact the study coordinator Kelly Mieszkalski, at (919) 681-4556, or toll free, at 1-866-292-7546.

Borderline Ovarian Cancer Counts!

As you may know, there has been some question concerning whether or not borderline ovarian cases should be considered malignant. In fact, for 2002, borderline ovarian cancer is not a reportable diagnosis to the North Carolina Central Cancer Registry (CCR.) **However, for rapid-case ascertainment purposes, the CCR has asked hospitals to include borderline ovarian cancer cases in their reporting.** The *North Carolina Ovarian Cancer Study* has always included borderline ovarian cancer as an eligible diagnosis, and we appreciate the extra effort cancer registrars have taken to send in borderline cases. Thank you for your continued cooperation!

Study Participation—How It Works

- The hospital Cancer Registrar sends monthly information on newly diagnosed ovarian cancer cases to the North Carolina Central Cancer Registry. (If needed, a representative from the Central Cancer Registry can assist with this task).
- The Central Cancer Registry forwards potentially eligible cases to the study project manager for determination of study eligibility.
- A consent form is sent to the attending physician requesting permission to contact their patient.
- When physician consent is received, a letter and brochure describing the study are sent to the patient.
- Shortly thereafter, a nurse-interviewer telephones the patient to discuss the study, determine eligibility, and, if eligible, invite her to participate.
- Hospitals are paid $10 for every eligible case reported to the NC Central Cancer Registry.

Progestin-Induced Apoptosis in the Macaque Ovarian Epithelium: Differential Regulation of Transforming Growth Factor-β

Gustavo C. Rodriguez, Nimesh P. Nagarsheth, Karen L. Lee, Rex C. Bentley, David K. Walmer, Mark Cline, Regina S. Whitaker, Pam Isner, Andrew Berchuck, Richard K. Dodge, Claude L. Hughes

Background: **Oral contraceptive (OC) use is associated with a reduced risk of ovarian cancer. An OC component, progestin, induces apoptosis in the primate ovarian epithelium. One regulator of apoptosis is transforming growth factor-β (TGF-β). We determined the effect of progestin on TGF-β expression in the primate ovarian epithelium and examined the relationship between TGF-β expression and apoptosis.** ***Methods:*** **Female cynomolgus macaques were randomly assigned to receive a diet for 35 months containing no hormones (n = 20); the OC Triphasil (n = 17); or each of its constituents, ethinyl estradiol (estrogen, n = 20) or levonorgestrel (progestin, n = 18), alone. Ovarian sections were immunostained with monoclonal antibodies against TGF-β1 or TGF-β2 plus TGF-β3 (TGF-β2/3) isoforms. The expression of TGF-β isoforms in four ovarian compartments (epithelium, oocytes, granulosa cells, and hilar vascular endothelium) was compared among treatment groups. The association between TGF-β expression and apoptosis, as determined by morphology and histochemistry, was examined in ovarian epithelium. All statistical tests were two-sided.** ***Results:*** **Compared with ovaries from the control and estrogen-only-treated monkeys, the ovaries of progestin-treated monkeys showed 1) a marked decrease in the expression of TGF-β1 and a concomitant increase in the expression of the TGF-β2/3 isoforms in the ovarian epithelium ($P<.001$), 2) an increase in the expression of TGF-β2/3 in the hilar vascular endothelium ($P<.001$), and 3) a marked decrease in TGF-β2/3 expression in granulosa cells ($P<.001$). The apoptotic index of the ovarian epithelium was highly associated with the change in expression from TGF-β1 ($P<.001$) to TGF-β2/3 ($P\leq.002$) induced by progestin treatment.** ***Conclusions:*** **Progestin induces differential regulation in the ovarian epithelium of TGF-β, a change in the expression of which is highly associated with apoptosis. These data suggest a possible biologic mechanism for the protective association between OC use and reduced ovarian cancer risk. [J Natl Cancer Inst 2002;94:50–60]**

Epithelial ovarian cancer remains an important public health problem. It is the fourth leading cause of cancer-related deaths among women in the United States and causes over 100 000 deaths annually worldwide *(1,2)*. Despite intensive research efforts over the past decade directed toward improved detection and treatment of ovarian cancer, the long-term survival of women with ovarian cancer has improved only modestly. Progress in the fight against ovarian cancer has been hampered by a number of factors, including late diagnosis, the molecular heterogeneity of ovarian tumors, the absence of highly curative chemotherapy, and the lack of a valid animal model for the disease.

The development of effective chemopreventive agents for ovarian cancer may represent our best hope for decreasing the ovarian cancer mortality rate in the future. A potent preventive agent already exists in the estrogen–progestin combination oral contraceptive (OC). Routine use of OCs for as little as 3 years confers as much as a 50% reduction in risk of ovarian cancer. The protective association increases with the duration of use and lasts for as long as 20 years after the discontinuation of use *(3–7)*. It has been our belief that, if the mechanism(s) underlying the remarkable protective effect of the OC can be elucidated, it may be possible to develop a pharmacologic chemopreventive strategy that is even more protective against ovarian cancer than OCs. Moreover, it may be possible to develop a chemopreventive strategy that is more broadly applicable than the use of OCs, potentially extending the benefits of chemoprevention beyond the reproductive age group to include those women who are menopausal, a group that currently lacks a nonsurgical approach for ovarian cancer prevention.

Although the biologic mechanism underlying the protective association between OC use and reduction in the risk of ovarian cancer remains unproven, two previously cited theories have focused on the known inhibitory effect of OCs on ovulation and on the inhibitory effect of OCs on the secretion of the pituitary gonadotropins follicle-stimulating hormone and luteinizing hormone. In the first theory, the inhibition of ovulation is presumed to reduce ovarian surface trauma and thereby to reduce the potential for genetic damage in the ovarian epithelium, while the second theory suggests that lowering gonadotropin levels potentially decreases a stimulus to proliferation in the ovary *(8–11)*. The ovulation-suppression theory has been challenged because the amount of risk reduction conferred by OCs far exceeds what would be predicted on the basis of the number of ovulations inhibited *(12)*. Similarly, the gonadotropin theory has been criticized because of the lack of evidence of an ovarian cancer-

Affiliations of authors: G. C. Rodriguez, R. S. Whitaker, P. Isner, A. Berchuck (Division of Gynecologic Oncology, Department of Obstetrics and Gynecology), N. P. Nagarsheth, K. L. Lee (Department of Obstetrics and Gynecology), R. C. Bentley (Department of Pathology), D. K. Walmer (Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology), R. K. Dodge (Department of Biostatistics, Duke Comprehensive Cancer Center), Duke University Medical Center, Durham, NC; M. Cline, Section of Comparative Medicine, Wake Forest University School of Medicine, Winston-Salem, NC; C. L. Hughes, Department of Obstetrics and Gynecology, Duke University Medical Center and Avalon Medical Group, Chapel Hill, NC.

Correspondence to: Gustavo C. Rodriguez, M.D., Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Rm. 1315, Evanston Northwestern Healthcare, 2650 Ridge Rd., Evanston, IL 60201 (e-mail: grodriguez@enh.org).

See "Notes" following "References."

protective effect associated with noncontraceptive estrogen use (which lowers gonadotropin levels) and because of the absence of an association between serum levels of follicle-stimulating hormone and luteinizing hormone and ovarian cancer risk *(12,13)*. Both of these theories fail to consider that the ovarian epithelium contains receptors for estrogen, progesterone, and androgen and that reproductive factors may affect ovarian cancer risk via a potent biologic interaction of sex steroid hormones with the ovarian epithelium *(14)*.

Recently, we performed a study in primates demonstrating that a combination estrogen–progestin OC has a potent apoptotic effect on the ovarian epithelium, mediated by the progestin component *(15)*. Primates randomly assigned in a 3-year trial to receive either combination estrogen and progestin or progestin alone had a fourfold to sixfold increase in the proportion of apoptotic ovarian epithelial cells as compared with control or estrogen-only-treated monkeys. The apoptosis pathway is one of the most important *in vivo* mechanisms that function to eliminate cells that have sustained DNA damage and, thus, are prone to malignant transformation *(16)*. In addition, a number of well-known chemopreventive agents have been demonstrated to activate the apoptosis pathway in the target tissues that they protect from neoplastic transformation *(17–32)*. The finding that progestins activate this critical pathway in the ovarian epithelium suggests that the protective effects afforded by OCs against ovarian cancer may at least in part be caused by progestin-mediated apoptosis. This forms the basis for an investigation of the progestin class of drugs as chemopreventive agents for epithelial ovarian cancer.

The regulation of apoptosis is complex and is influenced by numerous families of transcriptional factors, tumor suppressor genes, oncogenes, and growth factors *(33)*. Among the growth factors, transforming growth factor-β (TGF-β) has been implicated as an important regulator of apoptosis and as a mediator of the apoptotic effects of steroid hormones *(34–37)*. An association between the degree of TGF-β expression and apoptosis has been shown in cells derived from the breast *(38)* and prostate *(39)*, and the apoptotic activity of hormones such as the retinoids has been shown to be mediated at least in part by the activity of TGF-β *(18,19,40)*. Notably, multiple members of the steroid hormone superfamily, including the retinoids, vitamin D, and sex steroids, have been shown to modulate the expression of TGF-β, and the promoter region for specific TGF-β isotypes such as TGF-β2 and TGF-β3 contains response elements suggesting hormonal and developmental regulation *(41–51)*.

Given the link between TGF-β molecular pathways and apoptosis and evidence suggesting unique regulation of TGF-β by steroid hormones, we sought to determine in the current study whether there is an association between progestin-induced apoptotic effects in the primate ovarian epithelium and expression of TGF-β.

Materials and Methods

Animals/Randomization

As described previously *(15)*, 130 young adult female cynomolgus macaques (*Macaca fascicularis*), with an average age of 4.75 years, were randomly assigned into a study designed to evaluate the long-term biologic effects of the contraceptive Triphasil (Wyeth Ayerst, St. Davids, PA). The cynomolgus macaque is an excellent animal model for yielding experimental results that are pertinent to human reproductive biology. This nonhuman primate has a 28-day menstrual cycle that is similar to that of humans *(52–54)*. The study was a prospective, randomized, controlled trial designed for the primary endpoint of evaluating the effects of Triphasil and its individual components (ethinyl estradiol and levonorgestrel) on the cardiovascular system. Secondary outcomes to be analyzed included the biologic effects of Triphasil on the reproductive organs and breast. The randomization process was based on the serum lipid responses (total plasma cholesterol, triglycerides, and high-density lipoprotein-C) of animals to challenge with an atherogenic diet (44% of calories from fat, 0.28 mg of cholesterol per kilocalorie). After randomization, there were no differences between study groups with regard to body weight or age.

Forty of the animals were killed early in the study for baseline cardiovascular and lipoprotein studies, and an additional 14 animals died during the course of the study, primarily from trauma and diarrheal diseases. One animal was excluded because its ovarian tissue was not available for study. The remaining 75 animals were necropsied at the completion of the thirty-fifth month of the study and form the basis for this investigation. The study was approved by the Animal Care and Use Committee at the Wake Forest University School of Medicine, Winston-Salem, NC.

The macaques were prospectively randomly assigned via the lipid response parameters noted above into four groups to receive a diet for 35 months that contained 1) no hormones (control); 2) the oral combination contraceptive Triphasil, which is composed of estrogen (ethinyl estradiol) and progestin (levonorgestrel); 3) the estrogenic component of Triphasil (ethinyl estradiol) alone; or 4) the progestin component of Triphasil (levonorgestrel) alone. Hormones in the latter two groups were administered in the same dosage and schedule that occurs in a typical Triphasil regimen. Doses were scaled on the basis of caloric intake, which takes into account species differences in metabolic rate; this is the generally accepted way to achieve dosages comparable to those in women. The human-equivalent doses were given as follows: 6 days of 0.030 mg ethinyl estradiol plus 0.050 mg levonorgestrel per day, followed by 5 days of 0.040 mg ethinyl estradiol plus 0.075 mg levonorgestrel per day, followed by 10 days of 0.030 mg ethinyl estradiol plus 0.125 mg levonorgestrel per day, followed by 7 days of no hormone treatment. This cyclic regimen was repeated every 28 days continuously for 35 months. During the third week of the last month of the study, the animals were killed and their ovaries were carefully removed and preserved.

Tissue Preparation and Immunohistochemistry

From each animal in the study, one ovary was flash frozen by immersion in liquid nitrogen and saved for future molecular studies, and the other was formalin fixed and paraffin embedded.

Apoptosis. The median proportion of apoptotic ovarian epithelial cells associated with each treatment group had been quantified previously *(15)*. Briefly, 5-μm sections taken from the middle of each paraffin-embedded ovary were mounted on charged slides, and the ovarian epithelium was examined for morphologic and immunohistochemical evidence of apoptosis after staining with the APOPTAG-plus kit (Oncor, Gaithersburg, MD). Dark-brown, nuclear staining easily identified cells undergoing apoptosis. Tonsillar and deoxyribonuclease-digested tissue sections were used as positive controls. To calculate the

percentage of ovarian epithelial cells undergoing apoptosis, we counted both the total number of ovarian epithelial cells and the number undergoing apoptosis on each 5-μm section. The median proportion of cells undergoing apoptosis was calculated for each treatment group. At each step in this study, including the histologic examinations of the ovaries, the investigators were blinded with regard to the treatment group associated with each ovary.

TGF-β expression. Immunohistochemical expression of TGF-β was performed as previously described, with slight modification *(55)*. Briefly, 5-μm sections taken from the middle of each paraffin-embedded ovary were cut and mounted on charged slides. Two slides from each specimen were placed in a 60 °C oven for 1 hour. One slide was used as the negative control, while the other was used as the study specimen. The sections were deparaffinized, immersed in 0.3% hydrogen peroxide to quench endogenous peroxidase, hydrated, placed in Antigen Retrieval Citra solution at pH 6.0 (BioGenex Laboratories, Inc., San Ramon, CA), and then heated with an electric pressure cooker (Biocare Medical,Walnut Creek, CA) for 5 minutes. The sections were then cooled and rinsed with three washes of phosphate-buffered saline, preincubated in Power Block (BioGenex Laboratories, Inc.) for 10 minutes, and then incubated for 18 hours (overnight) at 4 °C in a humid chamber with primary antibody. For TGF-β1 expression, sections were immunostained with a monoclonal antibody that reacts with TGF-β1 but not TGF-β2 or TGF-β3 (2.5 μg/mL anti-TGF-β1 monoclonal antibody, catalog No. MAB 240; Research and Development Systems (Minneapolis, MN). To evaluate TGF-β2 and TGF-β3 (TGF-β2/3) expression, we stained sections with a mouse monoclonal antibody that reacts with the N-terminal region of both TGF-β2 and TGF-β3 but has no cross-reactivity with TGF-β1 (0.25 μg/mL TGF-β3 mouse monoclonal antibody; Oncogene Research Products, Cambridge, MA). For negative control specimens for TGF-β1 and TGF-β2/3 staining, mouse immunoglobulin G antibody (Coulter Corporation, Miami, FL) was applied at concentrations of 2.5 and 0.25 μg/mL, respectively. Slides were then washed three times with phosphate-buffered saline for 5 minutes each. Application of a biotinylated secondary antibody (Multi-Link Super Sensitive Detection System; BioGenex Laboratories) was performed at room temperature in a humid chamber for 20 minutes, then followed by three washes in phosphate-buffered saline for 5 minutes each. Peroxidase-conjugated streptavidin (Multi-Link Super Sensitive Detection System) was applied to sections and allowed to incubate for 20 minutes in a humid chamber, then followed by three washes in phosphate-buffered saline for 5 minutes each. Slides were incubated with freshly prepared 3,3-diaminobenzidine (D5637; Sigma Chemical Co., St. Louis, MO) chromogen solution (0.5% 3,3-diaminobenzidine, 0.6% hydrogen peroxide, and 0.05% Tris buffer) for 4 minutes and then washed in deionized water for 5 minutes to stop the reaction. This was followed by a 5-minute incubation in a 0.1 *M* sodium acetate solution and then staining with 1.5% methyl green for 5 minutes. Sections were dipped 10 times each in a serial fashion in the following solutions: 95% acetone, 95% acetone, 100% acetone, 100% acetone, 100% xylene, 100% xylene, and 100% xylene; then coverslips were placed on the slides. Umbilical cord sections, stained in a similar fashion, were used as positive control *(56)*.

The ovarian sections were examined by two independent sets of reviewers, all of whom were blinded to the hormone administration data (R. C. Bentley and K. L. Lee for TGF-β1 staining; R. C. Bentley and N. P. Nagarsheth for TGF-β2/3 staining). Staining for TGF-β was evaluated in four separate ovarian compartments of each study slide (ovarian surface epithelium, primordial oocyte cytoplasm, granulosa cells of tertiary follicles, and endothelium in ovarian hilar vessels) and graded according to the degree of staining intensity from 0 to 3+ (TGF-β1) and from 0 to 4+ (TGF-β2/3). High expression of TGF-β1 was defined by the slide reviewers as 2+ to 3+ staining intensity, whereas high expression of TGF-β2/3 was defined as 3+ to 4+ staining intensity. Three ovarian sections in the TGF-β1 staining group and two ovarian sections in the TGF-β2/3 staining group were excluded from grading because the samples were technically insufficient for evaluation.

Statistical Analysis

Quantitation and comparison of the median proportion of apoptotic cells in the ovarian epithelium had been performed previously *(15)*. Briefly, the Kruskal–Wallis test was used to perform multiple comparisons of all paired treatments *(57)*, and the statistical analysis was carried out with the use of the BMDP statistical software package (Biomathematical Data Package Statistical Software, Inc., Los Angeles, CA) *(58)*. For this study, the association between expression of the TGF-β isoforms and treatment was analyzed with the use of an overall approximate exact test for contingency tables *(59)*. In addition, each 2 × 2 table involving treatment and control was analyzed by use of Fisher's two-sided exact test. The relationship between treatment, amount of expression of TGF-β in the ovarian epithelium, and the mean proportion of apoptotic ovarian epithelial cells was analyzed by use of the general linear model (PROC GLM in the SAS statistical package; SAS Institute, Cary, NC) *(60)*. Multiple comparisons were performed with the use of Dunnett's two-sided test for each treatment compared with the control. The relationship between the proportion of high TGF-β expression and the mean proportion of apoptotic cells across treatments was analyzed by use of standard correlation analysis. The association between the TGF-β isoforms with respect to overexpression was analyzed with the use of the κ statistic *(61)*. All statistical tests were two-sided.

Results

Effect of Hormone Treatment on Expression of TGF-β

In general, in ovarian sections from the control group of monkeys, the pattern of expression of TGF-β was qualitatively similar to the pattern described previously in the human ovary *(62–65)*. In untreated monkeys (Fig. 1, A) and in estrogen-treated monkeys (Fig. 1, B), TGF-β1 expression was abundant in the ovarian epithelium and low to moderate in the stroma (structural tissue under epithelium) and the oocyte cytoplasm. Exposure to progestin either with estrogen (Fig. 1, C) or alone (Fig. 1, D) was associated with a marked decrease in the expression of TGF-β1 in the ovarian epithelium and in the oocyte compartment (*see* arrows in Fig. 1, B and D). The endothelial cells of the vascular hilum had little detectable expression of TGF-β1 (data not shown). Panels E–H in Fig. 1 represent the staining controls for monkey ovaries from four treatments, respectively.

The pattern of expression of TGF-β2/3 in untreated monkey ovaries was distinctly different from that of TGF-β1. Expression

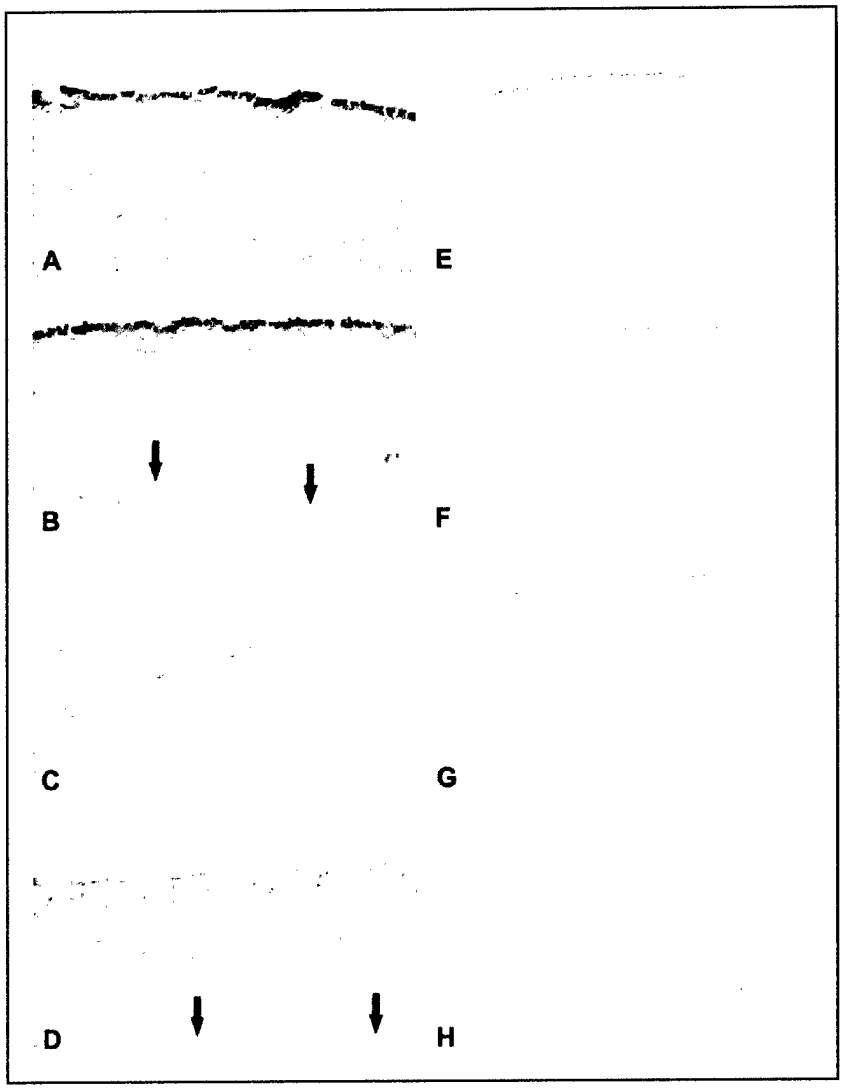


Fig. 1. Representative sections (original magnification ×80) of ovaries from macaques receiving four different hormone treatments were immunostained with anti-transforming growth factor (TGF)-β1 antibody (**A** = control [no treatment]; **B** = ethinyl estradiol alone; **C** = ethinyl estradiol plus levonorgestrel; **D** = levonorgestrel alone). TGF-β1 expression is abundant in the surface layer of ovarian epithelial cells in control (**A**) and estrogen-only-treated monkeys (**B**), and expression was markedly decreased in the progestin-treated monkeys (**C, D**). Progestin treatment (**D**) compared to estrogen treatment (**B**) was also associated with decreased expression of TGF-β1 in the oocyte compartment (*see arrows*). Negative controls for **A–D** stained with isotype-matched nonspecific mouse immunoglobulin G are shown in **E–H**, respectively.

of TGF-β2/3 was absent to scant in the ovarian epithelium, was high in the primordial oocyte cytoplasm, and was high in granulosa cells in large developing follicles (Fig. 2, A and B). Ovaries from estrogen-treated monkeys showed similar expression of TGF-β2/3 in epithelium and oocyte compartment (Fig. 2, D and E). Panels C and F in Fig. 2 are the respective staining controls for ovaries from control and estrogen-treated monkeys.

Weak signals for TGF-β2/3 expression were detected in endothelial cells in the ovarian hilum (Fig. 3, A) of untreated monkeys. The estrogen treatment resulted in practically no change (Fig. 3, B) in this expression. However, progestin treatment when either given in combination with estrogen (Fig. 3, C) or alone (Fig. 3, D) was associated with a marked increase in the expression of TGF-β2/3 in endothelial cells. The progestin treatment with or without estrogen also was associated with a marked increase in the expression of TGF-β2/3 in the ovarian surface epithelium but a decreased expression in granulosa cells in large, developing follicles (Fig. 4, A and B, and D and E, respectively, and asterisks Figs. 2, D, 4, A, and 4, D). Panels C and F in Fig. 4 were the staining controls for Fig. 4, A and B, and for Fig. 4, D and E, respectively.

The pattern of expression of TGF-β in the ovaries of primates receiving estrogen alone was similar to that in the control group (*see* Fig. 1, A, versus 1, B, for TGF-β1 in surface epithelium; Fig. 2, A and B, versus Fig. 2, D and E, for TGF-β2/3 in primordial oocytes and granulosa cells, and Fig. 3, A, versus 3, B, for TGF-β2/3 in hilar endothelial cells), suggesting that estrogen does not regulate expression of TGF-β in the ovary.

Effect of Hormone Treatment on Apoptosis in Ovarian Epithelium

In general, few apoptotic cells were noted in the ovarian epithelium from either the control or estrogen-only-treated monkeys (Fig. 5, A and B). In contrast, in progestin-treated monkeys, either those treated with combination ethinyl estradiol and levonorgestrel or with levonorgestrel alone, the ovarian epithelium contained numerous brown-staining apoptotic cells (Fig. 5, C and D). Additional morphologic findings in the ovarian epithe-

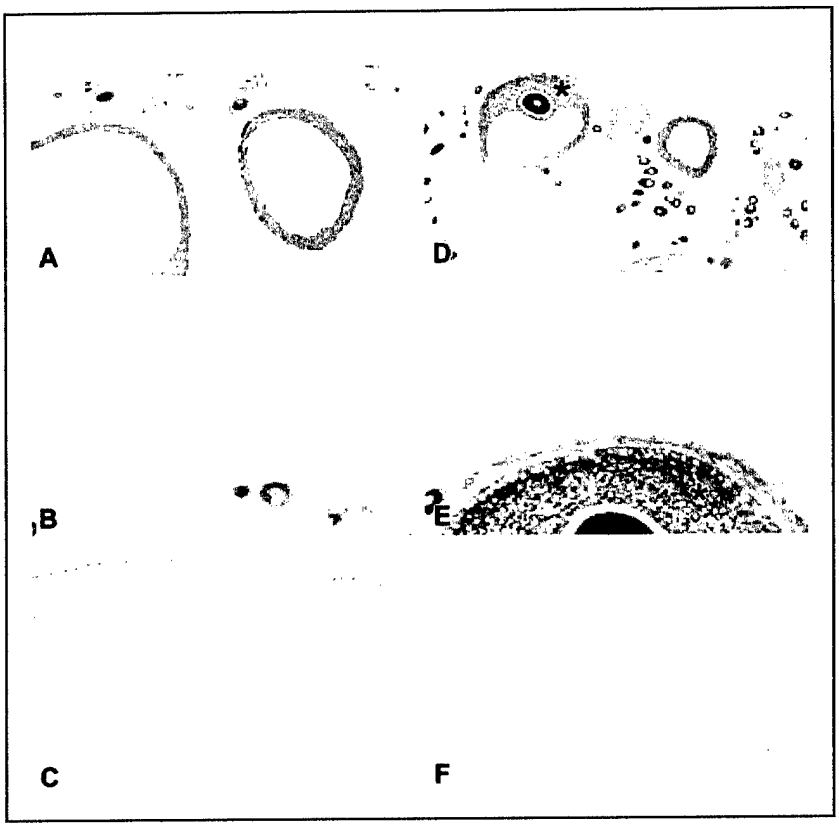


Fig. 2. Representative ovarian sections from control (no treatment) (**A**, original magnification ×25; **B**, original magnification ×80) and ethinyl estradiol-only treated (**D**, original magnification ×25; **E**, original magnification ×80) macaques immunostained with anti-transforming growth factor (TGF)-β2/3 antibody showing marked expression of TGF-β2/3 in primordial oocytes and granulosa cells (*) in large, developing follicles and little detectable expression of TGF-β2/3 in the ovarian surface epithelial layer. **C** and **F:** negative controls for **A** and **B** and **C** and **D**, respectively, stained with isotype-matched nonspecific mouse immunoglobulin G.

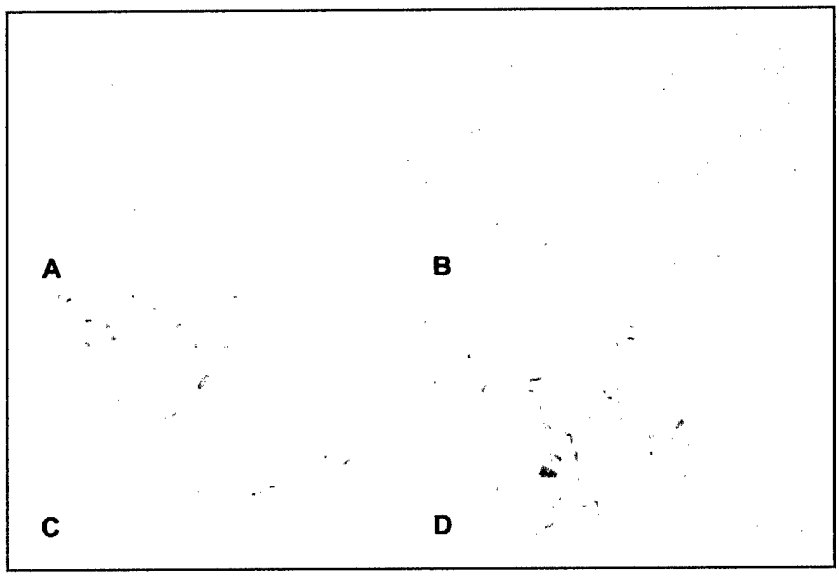


Fig. 3. Representative sections of the macaque ovarian hilum from the four hormone treatment groups immunostained with anti-transforming growth factor (TGF)-β2/3 antibody (**A** = control; **B** = ethinyl estradiol alone; **C** = ethinyl estradiol plus levonorgestrel; **D** = levonorgestrel alone). **C** and **D:** progestin treatment associated with a marked increase in expression of TGF-β2/3 in endothelial cells.

lium of progestin-treated monkeys included patches of ovarian surface devoid of epithelium, epithelial cells with sparse cytoplasm that appeared to be detaching from the surface, areas of epithelial denudation, and brown-staining apoptotic cells containing apoptotic bodies. The apoptotic changes noted in the ovarian epithelium of progestin-treated monkeys were not associated with any change in the proliferative index of the ovarian epithelium via staining for Ki-67 (data not shown).

Semiquantitative Determination of TGF-β Expression

Tables 1–3 summarize semiquantitative measurements of the hormonal regulation of TGF-β1 and TGF-β2/3 expression *in*

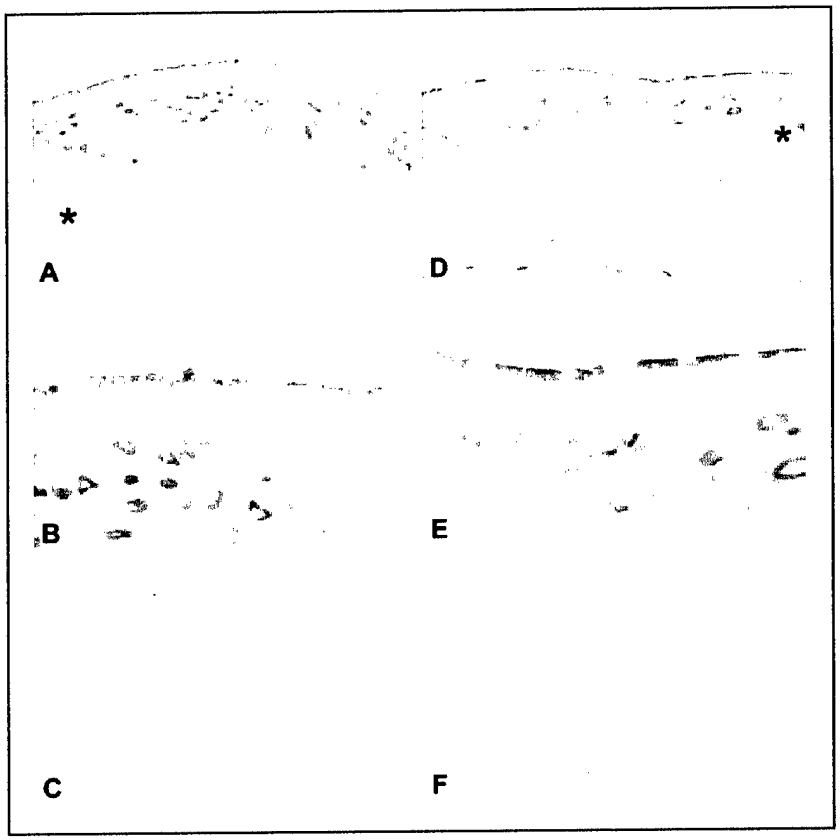


Fig. 4. Representative ovarian sections from combination estrogen–progestin-treated (**A,** original magnification ×25; **B,** original magnification ×80) and levonorgestrel-only treated (**D,** original magnification ×25; **E,** original magnification ×80) macaques immunostained with anti-transforming growth factor (TGF)-β2/3 antibody showing marked expression of TGF-β2/3 in the ovarian surface epithelium and decreased expression of TGF-β2/3 in granulosa cells (*) in large developing follicles. **C** and **F:** negative controls for **A** and **B** and **D** and **E,** respectively, stained with isotype-matched nonspecific mouse immunoglobulin G.

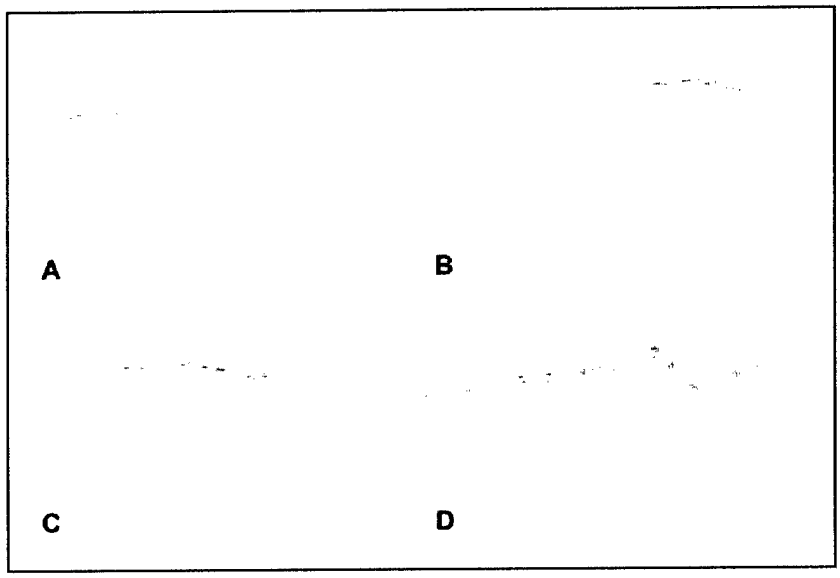


Fig. 5. Apoptag staining of representative macaque ovarian sections from the four hormone treatment groups (**A** = control; **B** = ethinyl estradiol alone; **C** = ethinyl estradiol plus levonorgestrel; **D** = levonorgestrel alone) showing marked apoptosis in the ovarian epithelium associated with progestin treatment (**C, D**) (original magnification ×80).

vivo. Progestin treatment, either combined with estrogen (Triphasil group) or administered alone (levonorgestrel group), was associated with a striking and highly statistically significant decrease in the expression of TGF-β1 in the ovarian epithelium ($P<.001$) and a moderate decrease in the expression of TGF-β1 in the oocyte cytoplasm ($P = .002$) (Table 1). In contrast, progestin treatment was associated with a marked increase in the expression of TGF-β2/3 in the ovarian epithelium ($P<.001$) (Table 2). Without exception, TGF-β2/3 expression in the ovarian epithelium was high (3+ to 4+ staining) in every monkey on

Table 1. Hormonal regulation of expression of transforming growth factor (TGF)-β1 in the macaque ovary

Treatment group	No. (%) ovaries/treatment group with high TGF-β1 expression (2+ to 3+) in each ovarian compartment				
	No.	Epithelium	Granulosa cells	Oocytes	Endothelium
Control	20	18 (90%)	7 (35%)	7 (35%)	0 (0%)
Ethinyl estradiol	19	16 (84%)	4 (21%)	2 (13%)	0 (0%)
Triphasil	16	3 (19%)	2 (13%)	0 (0%)	0 (0%)
Levonorgestrel	17	1 (6%)	2 (12%)	0 (0%)	0 (0%)
Overall approximate exact test		$P<.001$	$P = .31$	$P = .002$	$P = 1.00$
Control vs. ethinyl estradiol		$P = .66$	$P = .48$	$P = .13$	$P = 1.00$
Control vs. Triphasil		$P<.001$	$P = .24$	$P = .01$	$P = 1.00$
Control vs. levonorgestrel		$P<.001$	$P = .14$	$P = .009$	$P = 1.00$

Table 2. Hormonal regulation of expression of transforming growth factor (TGF)-β2/3 in the macaque ovary

Treatment group	No. (%) ovaries/treatment group with high TGF-β2/3 expression (3+ to 4+) in each ovarian compartment				
	No.	Epithelium	Granulosa cells	Oocytes	Endothelium
Control	19	6 (32%)	12 (63%)	14 (74%)	5 (26%)
Ethinyl estradiol	20	2 (10%)	8 (38%)	17 (81%)	3 (23%)
Triphasil	17	17 (100%)	1 (6%)	16 (94%)	16 (94%)
Levonorgestrel	17	17 (100%)	1 (6%)	14 (82%)	16 (94%)
Overall approximate exact test		$P<.001$	$P<.001$	$P = 0.45$	$P<.001$
Control vs. ethinyl estradiol		$P = .13$	$P = .11$	$P = .72$	$P = .72$
Control vs. Triphasil		$P<.001$	$P<.001$	$P = .18$	$P<.001$
Control vs. levonorgestrel		$P<.001$	$P<.001$	$P = .70$	$P<.001$

Table 3. Relationship between treatment, transforming growth factor (TGF)-β expression, and apoptosis in the macaque ovarian epithelium

Treatment	TGF-β1			TGF-β2/3		
	No.	% 2+ to 3+	Mean proportion of apoptotic cells in ovarian epithelium (95% CI*)	No.	% 3+ to 4+	Mean proportion of apoptotic cells in ovarian epithelium (95% CI*)
Control	20	90	6.3 (3.0 to 9.6)	19	32	6.4 (2.8 to 10)
Ethinyl estradiol	19	84	6.2 (1.8 to 10.6)	20	10	4.5 (1.2 to 7.8)
Triphasil	16	19	22.3 (13.6 to 31.0)†	17	100	21.2 (12.7 to 29.7)‡
Levonorgestrel	17	6	25.1 (16.0 to 34.2)†	17	100	26.4 (17.7 to 35.1)†

*CI = confidence interval for the mean.

†$P<.001$ by Dunnett's test comparing mean apoptotic index seen after treatment with that seen in controls (no treatment).

‡$P = .002$ by Dunnett's test comparing mean apoptotic index seen after treatment with that seen in controls (no treatment).

progestin (n = 34). Similarly, there was a significant increase in TGF-β2/3 expression in the ovarian hilar endothelial cells in monkeys on progestin ($P<.001$). In contrast, progestin treatment was associated with a marked decrease in TGF-β2/3 expression in granulosa cells ($P<.001$) (Table 2).

Within the ovarian epithelial compartment, comparison of the apoptotic index with the degree of change in the expression of the TGF-β isoforms revealed a highly significant correlation between changes in TGF-β expression and apoptosis ($P<.001$) (Table 3). With the use of the general linear model, for TGF-β1 there was a statistically significant treatment effect ($P<.001$) with respect to the mean proportion of apoptotic cells. Table 3 gives the mean proportion of apoptotic cells for each treatment group and shows that the Triphasil and levonorgestrel groups differ significantly from the control group ($P<.001$ for both comparisons with the use of Dunnett's test). Similarly, for TGF-β2/3 there was also a statistically significant treatment effect ($P<.001$), and Table 3 shows that the Triphasil and levonorgestrel groups differ statistically significantly from the control group ($P = .002$, and $P<.001$, respectively, by Dunnett's test). The Pearson correlation coefficients between the proportion of high TGF-β expression and the mean proportion of apoptotic cells across treatments were –0.998 ($P = .002$) for TGF-β1 and 0.973 ($P = .03$) for TGF-β2/3. Finally, overall, there was a negative association between TGF-β2/3 overexpression and TGF-β1 overexpression ($\kappa = -0.62$; $P<.001$). Taken together, these data demonstrate the novel finding that progestin-induced apoptosis in the ovarian epithelium is associated with an isoform switch in expression of TGF-β.

Discussion

To the best of our knowledge, this is the first study to demonstrate regulation of TGF-β expression in the primate ovarian epithelium *in vivo* by a contraceptive steroid. We found TGF-β expression to be differentially regulated in the ovarian epithelium of primates that received progestin, administered either in the form of an estrogen–progestin combination pill or alone. Progestin treatment was associated with a marked decrease in expression of TGF-β1 concomitant with a marked increase in expression of TGF-β2/3. In addition, the progestin-induced change in TGF-β isoform expression was highly correlated with

an increase in apoptosis in the ovarian epithelium. Estrogen treatment appeared to have no impact on TGF-β expression in the ovary.

The mechanism underlying progestin regulation of TGF-β expression in the ovary remains to be determined. It is possible that progestins induce factors in the ovarian stroma that regulate TGF-β pathways at sites throughout the ovary via a paracrine effect. Conversely, it is possible that progestins act directly through classic progesterone receptor-mediated pathways to effect TGF-β expression. The results of this study suggest that progestin regulation of ovarian TGF-β expression occurs via a direct effect in that changes in TGF-β expression associated with progestin treatment were primarily localized to sites in the ovary known to express progestin receptors. These include the ovarian epithelium *(66)* and granulosa cells of large follicles *(67)*. In addition, the finding that progestins increase the expression of TGF-β2/3 in the ovarian epithelium while at the same time decreasing expression of TGF-β2/3 in granulosa cells supports the notion not only that progestin induction of TGF-β2/3 in the ovarian epithelium is a direct effect but also that the end effect of progestins in the ovary is site specific. It is interesting that we also noted increased expression of TGF-β2/3 in the endothelial cells of the vascular hilum in progestin-treated monkeys. It has been shown recently that endothelial cells contain functional progesterone receptors and that progesterone inhibits endothelial proliferation *(68)*.

There is mounting evidence that differential regulation of peptide growth factors by steroid hormones contributes to the diverse end effects of these hormones in target tissues. Among the growth factors, TGF-β has been shown to be differentially regulated by estrogens, retinoids, androgens, and vitamin D compounds. In bone, raloxifene increases the expression of TGF-β3 while having no effect on the expression of TGF-β1 and TGF-β2 *(69)*. In cells derived from the breast, estradiol decreases the expression of TGF-β2 and TGF-β3 while having no effect on the expression of TGF-β1 *(70)*, whereas tamoxifen has been shown to increase the expression of TGF-β1 *(71)*. In chondrocytes, vitamin D increases the expression of TGF-β2 and decreases the expression of TGF-β1 and TGF-β3 *(72)*. Glucocorticoids differentially regulate TGF-β in healing wounds, leading to the suppression of TGF-β1 and TGF-β2 and the increased expression of TGF-β3 *(73)*. In the palates of mice, retinoids have been shown to decrease the expression of TGF-β1 while having no effect on the expression of other TGF-β isoforms *(74)*, whereas in keratinocytes, induction of TGF-β2 is a major mechanism underlying the biologic effects of retinoids *(51,75)*. Finally, in the male accessory organs, androgen withdrawal is associated with both apoptosis and differential regulation of TGF-β *(76)*. Thus, the TGF-β isotypes appear to be differentially regulated in a tissue-specific manner. Although the mechanism underlying the complex regulation of TGF-β by hormones is not completely understood, differences in the promoter region among the TGF-β isoforms or in post-transcriptional events may be means by which TGF-β is differentially regulated *(77–80)*.

Although the design of our study does not allow us to prove the causal relationship between TGF-β expression and apoptosis, the finding that changes in TGF-β expression were highly associated with apoptosis in the ovarian epithelium in primates on progestin is strongly supportive of the hypothesis that progestin-induced apoptosis may be occurring via a TGF-β-mediated molecular pathway. In addition to the findings of this study, other lines of evidence support this hypothesis. First, in hormone-responsive tissues, such as the breast and prostate, TGF-β has been shown to mediate the apoptotic effects of steroid hormones, including the antiestrogens, retinoids, and vitamin D *(40,81,82)*. Second, in tissues that are progesterone receptor positive, such as the breast and endometrium, progestins have been shown to be associated with both induction of TGF-β and apoptosis *(37,41,83–88)*. Third, both our group and others *(89–91)* have shown previously that some ovarian cancer cell lines undergo apoptosis when treated with TGF-β. It is interesting that, in our laboratory, we were not able to demonstrate induction of apoptosis in normal ovarian epithelial cells treated with TGF-β. It is possible, however, that the assay techniques used in our study were not sufficiently sensitive to detect apoptosis in a limited sample of normal human ovarian epithelial cells. Alternatively, it is possible that our *in vitro* experiments lacked an important cofactor present *in vivo* that is required for TGF-β-mediated apoptosis to occur or that our *in vitro* conditions failed to simulate the complex interrelationship of TGF-β isoform expression required for apoptosis to occur in nonmalignant human ovarian epithelial cells. A fourth line of evidence is that TGF-β is related to müllerian inhibitory factor, a peptide that causes complete regression of the müllerian system (the precursor to the uterus, fallopian tubes, and upper vagina) in the developing male embryo *(92–95)*. In the embryo, the müllerian tract develops from an invagination of the celomic epithelium and, therefore, is derived from the same embryonic precursor tissue as the ovarian epithelium *(96)*. Given the marked inhibitory effect that the müllerian inhibitory factor has on the müllerian system, it is interesting to speculate that the ovarian epithelium may be uniquely susceptible *in vivo* to undergoing apoptosis in response to TGF-β and that agents that selectively regulate TGF-β in the ovarian epithelium may be potent apoptosis-inducing agents and cancer preventive agents.

A growing body of laboratory and animal evidence has implicated TGF-β as a potent tumor suppressor and cancer preventive agent *(97–99)*. Transgenic mice that have a constitutively active form of TGF-β1 are resistant to 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors *(100)*. Conversely, mice with heterozygous deletions of one copy of the TGF-β gene have an increased susceptibility to chemical carcinogenesis *(101)*. In humans, mutations have been described in the TGF-β signaling pathway in a variety of tumors, including cancers of colon, cancers of the gastric, pancreatic, and uterine systems, and cancers of lymphoid organs *(99)*. Furthermore, a number of cellular oncogenes are known to inhibit TGF-β activity. Finally, TGF-β has been implicated as a mediator of the biologic effects of a number of chemopreventive agents, including tamoxifen, which induces expression of TGF-β1 in stromal cells in the breast *(71)*, as well as retinoids, which induce TGF-β in the prostate and respiratory tract *(98)*. Taken together, these data provide compelling evidence that TGF-β plays an important role as an inhibitor of carcinogenesis.

In light of the known association between TGF-β and cancer prevention, the observation that OCs markedly alter expression of TGF-β in the ovarian epithelium implicates TGF-β as possibly mediating the ovarian cancer-protective effects of the pill. The finding that OCs induce both apoptosis and TGF-β in the ovary suggests that OCs may be acting as true chemopreventive agents by activating molecular pathways known to arrest or

reverse the process of carcinogenesis, rather than simply by limiting ovulation-induced damage in the ovarian epithelium. Moreover, the finding that activation of apoptosis and differential regulation of TGF-β are related specifically to the progestin component of the OC provides strong evidence in support of the notion that biologic effects produced by the progestin component may be major mechanisms underlying the marked protection conferred by OCs against ovarian cancer.

The discovery that contraceptive progestins activate cancer-preventive molecular pathways in the ovarian epithelium opens the door to the development of a highly effective pharmacologic preventive strategy for ovarian cancer that may be more effective and more broadly applicable than OCs. For example, if the protective effects of OCs were solely a result of ovulation inhibition as previously believed, then there is little potential for designing improved OC formulations that have enhanced ovarian cancer-protective effects, and the protective effects can only be beneficial for premenopausal women who are ovulating. However, if OCs confer marked ovarian cancer protection through a biologic effect unrelated to ovulation inhibition, then it may be possible to design OC formulations that maximize these biologic effects to achieve enhanced ovarian cancer protection. In addition, it may be possible to develop a pharmacologic preventive strategy that can be applied to all women, including administration of a pharmacologic regimen that has ovarian cancer-preventive effects in postmenopausal women who are anovulatory.

The ideal preventive agent for ovarian cancer may be composed of a combination of agents that act in an additive or synergistic fashion to maximally activate molecular pathways that inhibit carcinogenesis in the ovarian epithelium, thereby maximizing ovarian cancer prevention while minimizing side effects. In this regard, agents selected from the steroid hormone superfamily on the basis of their ability to activate TGF-β are uniquely attractive. Steroid hormones would be expected to specifically target only cells expressing appropriate steroid ligand receptor. In addition, given the short half-life of active TGF-β *in vivo,* rapid clearance of TGF-β at target sites would limit the systemic toxicity associated with chemoprevention *(75).* It is interesting to speculate that the combination of a progestin, which regulates TGF-β in the ovarian epithelium, and a retinoid and/or vitamin D might achieve synergistic or additive effects on TGF-β pathways in the ovarian epithelium, leading to a powerful cancer preventive agent. Synergistic effects on both growth inhibition and apoptosis have been described *in vitro* in cells derived from ovarian epithelium with the use of the combination of vitamin A derivatives and TGF-β *(102).* Similarly, cross-talk has been described between vitamin D and TGF-β signaling pathways, and the combination of vitamin D and TGF-β has been shown to have synergistic effects *in vitro (103,104).* These approaches will be the subject of further investigation as we work toward the development of optimal chemopreventive strategies.

References

(1) Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics, 1999. CA Cancer J Clin 1999;49:8–31.

(2) Parkin DM, Pisani P, Ferlay J. Global cancer statistics. CA Cancer J Clin 1999;49:33–64.

(3) Rosenberg L, Palmer JR, Zauber AG, Warshauer ME, Lewis JL Jr, Strom BL, et al. A case–control study of oral contraceptive use and invasive epithelial ovarian cancer. Am J Epidemiol 1994;139:654–61.

(4) Epithelial ovarian cancer and combined oral contraceptives: the WHO collaborative study of neoplasia and steroid contraceptives. Int J Epidemiol 1989;18:538–45.

(5) The reduction in risk of ovarian cancer associated with oral-contraceptive use. The Cancer and Steroid Hormone Study of the Centers for Disease Control and the National Institute of Child Health and Human Development. N Engl J Med 1987;316:650–5.

(6) Gross TP, Schlesselman JJ. The estimated effect of oral contraceptive use on the cumulative risk of epithelial ovarian cancer. Obstet Gynecol 1994; 83:419–24.

(7) Franceschi S, Parazzini F, Negri E, Booth M, La Vecchia C, Beral V, et al. Pooled analysis of 3 European case–control studies of epithelial ovarian cancer: III. Oral contraceptive use. Int J Cancer 1991;49:61–5.

(8) Wu ML, Whittemore AS, Paffenbarger RS Jr, Sarles DL, Kampert JB, Grosser S, et al. Personal and environmental characteristics related to epithelial ovarian cancer. I. Reproductive and menstrual events and oral contraceptive use. Am J Epidemiol 1988;128:1216–27.

(9) Greene MH, Clark JW, Blayney DW. The epidemiology of ovarian cancer. Semin Oncol 1984;11:209–26.

(10) Cramer DW, Welch WR. Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. J Natl Cancer Inst 1983;71:717–21.

(11) Whittemore AS, Harris R, Itnyre J. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 U.S. case–control studies. II. Invasive epithelial ovarian cancers in white women. Collaborative Ovarian Cancer Group. Am J Epidemiol 1992;136:1184–203.

(12) Risch HA, Weiss NS, Lyon JL, Daling JR, Liff JM. Events of reproductive life and the incidence of epithelial ovarian cancer. Am J Epidemiol 1983;117:128–39.

(13) Helzlsouer KJ, Alberg AJ, Gordon GB, Longcope C, Bush TL, Hoffman SC, et al. Serum gonadotropins and steroid hormones and the development of ovarian cancer. JAMA 1995;274:1926–30.

(14) Risch HA. Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. J Natl Cancer Inst 1998;90:1774–86.

(15) Rodriguez GC, Walmer DK, Cline M, Krigman H, Lessey BA, Whitaker RS, et al. Effect of progestin on the ovarian epithelium of macaques: cancer prevention through apoptosis? J Soc Gynecol Investig 1998;5: 271–6.

(16) Canman CE, Chen CY, Lee MH, Kastan MB. DNA damage responses: p53 induction, cell cycle pertubations, and apoptosis. Cold Spring Harb Symp Quant Biol 1994;59:277–86.

(17) Chan LN, Zhang S, Cloyd M, Chan TS. *N*-(4-Hydroxyphenyl) retinamide prevents development of T-lymphomas in AKR/J mice. Anticancer Res 1997;17:499–503.

(18) Ponzoni M, Bocca P, Chiesa V, Decensi A, Pistoia V, Raffaghello L, et al. Differential effects of *N*-(4-hydroxyphenyl)retinamide and retinoic acid on neuroblastoma cells: apoptosis versus differentiation. Cancer Res 1995;55:853–61.

(19) Delia D, Aiello A, Lombardi L, Pelicci PG, Grignani F, Grignani F, et al. *N*-(4-Hydroxyphenyl)retinamide induces apoptosis of malignant hemopoietic cell lines including those unresponsive to retinoic acid. Cancer Res 1993;53:6036–41.

(20) Seewaldt VL, Yim JR, Caldwell LE, Johnson BS, Swisshelm Y, Collins SJ. All-*trans*-retinoic acid mediates G_1 arrest but not apoptosis of normal human mammary epithelial cells. Cell Growth Differ 1995;6:863–9.

(21) Lotan R. Retinoids in cancer chemoprevention. FASEB J 1996;10: 1031–9.

(22) Sankaranarayanan R, Mathew B. Retinoids as cancer-preventive agents. IARC Sci Publ 1996;139:47–59.

(23) Toma S, Isnardi L, Raffo P, Dastoli G, De Francisci E, Riccardi L, et al. Effects of all-*trans*-retinoic acid and 13-*cis*-retinoic acid on breast-cancer cell lines: growth inhibition and apoptosis induction. Int J Cancer 1997; 70:619–27.

(24) Oridate N, Lotan D, Mitchell MF, Hong WK, Lotan R. Inhibition of proliferation and induction of apoptosis in cervical carcinoma cells by retinoids: implications for chemoprevention. J Cell Biochem Suppl 1995; 23:80–6.

(25) Dolivet G, Ton Van J, Sarini J, Wattel E, Lagarde P, Chomy F, et al. Current knowledge on the action of retinoids in carcinoma of the head and neck. Rev Laryngol Otol Rhinol (Bord) 1996;117:19–26.

(26) Kuo SM. Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells. Cancer Lett 1996;110:41–8.
(27) Thompson HJ, Jiang C, Lu J, Mehta RG, Piazza GA, Paranka NS, et al. Sulfone metabolite of sulindac inhibits mammary carcinogenesis. Cancer Res 1997;57:267–71.
(28) Reddy BS, Wang CX, Samaha H, Lubet R, Steele VE, Kelloff GJ, et al. Chemoprevention of colon carcinogenesis by dietary perillyl alcohol. Cancer Res 1997;57:420–5.
(29) Gould MN. Cancer chemoprevention and therapy by monoterpenes. Environ Health Perspect 1997;105 Suppl 4:977–9.
(30) Pascale RM, Simile MM, De Miglio MR, Nufris A, Daino L, Seddaiu MA, et al. Chemoprevention by *S*-adenosyl-L-methionine of rat liver carcinogenesis initiated by 1,2-dimethylhydrazine and promoted by orotic acid. Carcinogenesis 1995;16:427–30.
(31) Thompson HJ, Wilson A, Lu J, Singh M, Jiang C, Upadhyaya P, et al. Comparison of the effects of an organic and an inorganic form of selenium on a mammary carcinoma cell line. Carcinogenesis 1994;15:183–6.
(32) el-Bayoumy K, Upadhyaya P, Chae YH, Sohn OS, Rao CV, Fiala E, et al. Chemoprevention of cancer by organoselenium compounds. J Cell Biochem Suppl 1995;22:92–100.
(33) Franko J, Pomfy M, Prosbova T. Apoptosis and cell death (mechanisms, pharmacology and promise for the future). Acta Medica (Hradec Kralove) 2000;43:63–8.
(34) Haufel T, Dormann S, Hanusch J, Schwieger A, Bauer G. Three distinct roles for TGF-β during intercellular induction of apoptosis: a review. Anticancer Res 1999;19:105–12.
(35) Bursch W, Oberhammer F, Jirtle RL, Askari M, Sedivy R, Grasl-Kraupp B, et al. Transforming growth factor-β1 as a signal for induction of cell death by apoptosis. Br J Cancer 1993;67:531–6.
(36) Chiarugi V, Magnelli L, Cinelli M. Complex interplay among apoptosis factors: RB, p53, E2F, TGF-β, cell cycle inhibitors and the bcl2 gene family. Pharmacol Res 1997;35:257–61.
(37) Rotello RJ, Lieberman RC, Purchio AF, Gershenson LE. Coordinated regulation of apoptosis and cell proliferation by transforming growth factor β 1 in cultured uterine epithelial cells. Proc Natl Acad Sci U S A 1991;88:3412–5.
(38) Strange R, Li F, Saurer S, Burkhardt A, Friis RR. Apoptotic cell death and tissue remodeling during mouse mammary gland involution. Development 1992;115:49–58.
(39) Kyprianou N, Isaacs JT. Expression of transforming growth factor-beta in the rat ventral prostate during castration-induced programmed cell death. Mol Endocrinol 1989;3:1515–22.
(40) Roberson KM, Penland SN, Padilla GM, Selvan RS, Kim CS, Fine RL, et al. Fenretinide: induction of apoptosis and endogenous transforming growth factor β in PC-3 prostate cancer cells. Cell Growth Differ 1997; 8:101–11.
(41) Colletta AA, Wakefield LM, Howell FV, Danielpour D, Baum M, Sporn MB. The growth inhibition of human breast cancer cells by a novel synthetic progestin involves the induction of transforming growth factor beta. J Clin Invest 1991;87:277–83.
(42) Reiss M, Barcellos-Hoff MH. Transforming growth factor-β in breast cancer: a working hypothesis. Breast Cancer Res Treat 1997;45:81–95.
(43) Dannecker C, Possinger K, Classen S. Induction of TGF-β by an antiprogestin in the human breast cancer cell line T-47D. Ann Oncol 1996; 7:391–5.
(44) Lucia MS, Sporn MB, Roberts AB, Stewart LV, Danielpour D. The role of transforming growth factor-beta1, -beta2, and -beta3 in androgen-responsive growth of NRP-152 rat prostatic epithelial cells. J Cell Physiol 1998;175:184–92.
(45) Jeng MH, ten Dijke P, Iwata KK, Jordan VC. Regulation of the levels of three transforming growth factor β mRNAs by estrogen and their effects on the proliferation of human breast cancer cells. Mol Cell Endocrinol 1993;97:115–23.
(46) Heberden C, Denis I, Pointillart A, Mercier T. TGF-β and calcitriol. Gen Pharmacol 1998;30:145–51.
(47) Gold LI. The role for transforming growth factor-β (TGF-β) in human cancer. Crit Rev Oncog 1999;10:303–60.
(48) Wu Y, Craig TA, Lutz WH, Kumar R. Identification of 1 α 25-dihydroxyvitamin D3 response elements in the human transforming growth factor β2 gene. Biochemistry 1999;38: 2654–60.
(49) Roberts AB. Molecular and cell biology of TGF-β. Miner Electrolyte Metab 1998;24:111–9.
(50) Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R, et al. Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. Cell 1987; 48:417–28.
(51) Roberts AB, Sporn MB. Mechanistic interrelationships between two superfamilies: the steroid/retinoid receptors and transforming growth factor-β. Cancer Surv 1992;14:205–20.
(52) Brenner RM, Slayden OD. Cyclic changes in the primate oviduct and endometrium. In: Knobil E, Neill JD, editors. The physiology of reproduction. New York (NY): Raven Press; 1994. p. 541–69.
(53) Hotchkiss J, Knobil E. The menstrual cycle and its neuroendocrine control. In: Knobil E, Neill JD, editors. The physiology of reproduction. New York (NY): Raven Press; 1994. p. 711–36.
(54) Kaiserman-Abramof IR, Padykula HA. Ultrastructural epithelial zonation of the primate endometrium (rhesus monkey). Am J Anat 1989;184: 13–30.
(55) Hurteau J, Rodriguez GC, Whitaker RS, Shah S, Mills G, Bast RC, et al. Transforming growth factor-beta inhibits proliferation of human ovarian cancer cells obtained from ascites. Cancer 1994;74:93–9.
(56) Stewart AA, Haley JD, Qu GY, Stam K, Fenyo D, Chait BT, et al. Umbilical cord transforming growth factor-β3: isolation, comparison with recombinant TGF-β3 and cellular localization. Growth Factors 1996;13: 87–98.
(57) Hollander M, Wolfe DA. Nonparametric statistical methods. New York (NY): Wiley; 1973.
(58) BMDP statistical software manual. Los Angeles (CA): University of California Press; 1992. p. 459–61.
(59) Mehta CR, Patel NR. A network algorithm for performing Fisher's exact test in rxc contingency tables. J Am Stat Assoc 1983;78:427–34.
(60) SAS Institute Inc. SAS/STAT user's guide, version 6. Vol 2. 4th ed. Cary (NC): SAS Institute, Inc.; 1990.
(61) Agresti A. Categorical data analysis. New York (NY): Wiley; 1990.
(62) Henriksen R, Gobl A, Wilander E, Oberg K, Miyazono K, Funa K. Expression and prognostic significance of TGF-β isotypes, latent TGF-β1 binding protein, TGF-β type I and type II receptors, and endoglin in normal ovary and ovarian neoplasms. Lab Invest 1995;73:213–20.
(63) Schilling B, Yeh J. Expression of transforming growth factor (TGF)-β1, TGF-β2, and TGF-β3 and of type I and II TGF-β receptors during the development of the human fetal ovary. Fertil Steril 1999;72:147–53.
(64) Chegini N, Flanders KC. Presence of transforming growth factor-β and their selective cellular localization in human ovarian tissue of various reproductive stages. Endocrinology 1992;130:1707–15.
(65) Berchuck A, Rodriguez G, Olt G, Whitaker R, Boente MP, Arrick BA, et al. Regulation of growth of normal ovarian epithelial cells and ovarian cancer cell lines by transforming growth factor-beta. Am J Obstet Gynecol 1992;166:676–84.
(66) Lau KM, Mok SC, Ho SM. Expression of human estrogen receptor-α and -β, progesterone receptor, and androgen receptor mRNA in normal and malignant ovarian epithelial cells. Proc Natl Acad Sci U S A 1999;96: 5722–27.
(67) Revelli A, Pacchioni D, Cassoni P, Bussolati G, Massobrio M. *In situ* hybridization study of messenger RNA for estrogen receptor and immunohistochemical detection of estrogen and progesterone receptors in the human ovary. Gynecol Endocrinol 1996;10:177–86.
(68) Vazquez F, Rodriguez-Manzaneque JC, Lydon JP, Edwards DP, O'Malley BW, Iruela-Arispe ML. Progesterone regulates proliferation of endothelial cells. J Biol Chem 1999;274:2185–92.
(69) Yang NN, Bryant HU, Hardikar S, Sato M, Galvin RJ, Glasebrook AL, et al. Estrogen and raloxifene stimulate transforming growth factor-beta 3 gene expression in rat bone: a potential mechanism for estrogen of raloxifene-mediated bone maintenance. Endocrinology 1996;137:2075–84.
(70) Arrick BA, Kore M, Derynck R. Differential regulation of expression of three transforming growth factor beta species in human breast cancer cell lines by estradiol. Cancer Res 1990;50:299–303.
(71) Butta A, MacLennan K, Flanders KC, Sacks NP, Smith I, McKinna A, et al. Induction of transforming growth factor β1 in human breast cancer *in vivo* following tamoxifen treatment. Cancer Res 1992;52:4261–4.
(72) Farquharson C, Law AS, Seawright E, Burt DW, Whitehead CC. The

expression of transforming growth factor-beta by cultured chick growth plate chondrocytes: differential regulation by 1,25-dihydroxyvitamin D3. J Endocrinol 1996;149:277–85.

(73) Frank S, Madlener M, Werner S. Transforming growth factors beta1, beta2, and beta3 and their receptors are differentially regulated during normal and impaired wound healing. J Biol Chem 1996;271:10188–93.

(74) Degitz SJ, Morris D, Foley GL, Francis BM. Role of TGF-beta in RA-induced cleft palate in CD-1 mice. Teratology 1998;58:197–204.

(75) Wakefield L, Kim SJ, Glick A, Winokur T, Colletta A, Sporn M. Regulation of transforming growth factor-β subtypes by members of the streroid hormone superfamily. J Cell Sci Suppl 1990;13:139–48.

(76) Desai KV, Kondaiah P. Androgen ablation results in differential regulation of transforming growth factor-beta isoforms in rat male accessory sex organs and epididymis. J Mol Endocrinol 2000;24:253–60.

(77) Geiser AG, Busam KJ, Kim SJ, Lafyatis R, O'Reilly MA, Webbink R, et al. Regulation of the transforming growth factor-beta 1 and -beta 3 promoters by transcription factor Sp1. Gene 1993;129:223–8.

(78) Kim SJ, Park K, Koeller D, Kim KY, Wakefield LM, Sporn MB, et al. Post-transcriptional regulation of the human transforming growth factor-beta gene. J Biol Chem 1992;267:13702–7.

(79) Kim SJ, Glick A, Sporn MB, Roberts AB. Characterization of the promoter region of the human transforming growth factor-beta 1 gene. J Biol Chem 1989;264:402–8.

(80) Noma T, Glick AB, Geiser AG, O'Reilly MA, Miller J, Roberts AB, et al. Molecular cloning and structure of the human transforming growth factor-beta 2 gene promoter. Growth Factors 1991;4:247–55.

(81) El Etreby MF, Liang Y, Wrenn RW, Schoenlein PV. Additive effect of mifepristone and tamoxifen on apoptotic pathways in MCF-7 human breast cancer cells. Breast Cancer Res Treat 1998;51:149–68.

(82) James SY, Mackay AG, Colston KW. Effects of 1,25 dihydroxyvitamin D_3 and its analogues on induction of apoptosis in breast cancer cells. J Steroid Biochem Mol Biol 1996;58:395–401.

(83) Formby B, Wiley TS. Progesterone inhibits growth and induces apoptosis in breast cancer cells: inverse effects on Bcl-2 and p53. Ann Clin Lab Sci 1998;28:360–9.

(84) Bruner KL, Eisenberg E, Gorstein F, Osteen KG. Progesterone and transforming growth factor-beta coordinately regulate suppression of endometrial matrix metalloproteinases in a model of experimental endometriosis. Steroids 1999;64:648–53.

(85) Casslen B, Sandberg T, Gustavsson B, Willen R, Nilbert M. Transforming growth factor β1 in the human endometrium. Cyclic variation, increased expression by estradiol and progesterone, and regulation of plasminogen activators and plasminogen activator inhibitor-1. Biol Reprod 1998;58:1343–50.

(86) Ace CI, Okulicz WC. Differential gene regulation by estrogen and progesterone in the primate endometrium. Mol Cell Endocrinol 1995;115:95–103.

(87) Amezcua CA, Zheng W, Muderspach LI, Felix JC. Down-regulation of bcl-2 is a potential marker of the efficacy of progestin therapy in the treatment of endometrial hyperplasia. Gynecol Oncol 1999;73:126–36.

(88) Amezcua CA, Lu JJ, Felix JC, Stanczyk FZ, Zheng W. Apoptosis may be an early event of progestin therapy for endometrial hyperplasia. Gynecol Oncol 2000;79:169–76.

(89) Havrilesky LJ, Hurteau JA, Whitaker RS, Elbendary A, Wu S, Rodriguez GC, et al. Regulation of apoptosis in normal and malignant ovarian epithelial cells by transforming growth factor β. Cancer Res 1995;55:944–8.

(90) Lafon C, Mathieu C, Guerrin M, Pierre O, Vidal S, Vallette A. Transforming growth factor β1-induced apoptosis in human ovarian carcinoma cells: protection by the antioxidant *N*-acetylcysteine and bcl-2. Cell Growth Differ 1996;7:1095–104.

(91) Mathieu C, Jozan S, Mazars P, Côme MG, Moisand A, Vallette A. Density-dependent induction of apoptosis by transforming growth factor-β1 in a human ovarian carcinoma cell line. Exp Cell Res 1995;216:13–20.

(92) Mishina Y, Whitworth DJ, Racine C, Behringer RR. High specificity of Müllerian-inhibiting substance signaling *in vivo*. Endocrinology 1999;140:2084–8.

(93) Voutilainen R, Miller WL. Potential relevance of mullerian-inhibiting substance to ovarian physiology. Semin Reprod Endocrinol 1989;7:88–93.

(94) Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, Cheung A, et al. Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. Cell 1986;45:685–98.

(95) Byskov AG. Differentiation of mammalian embryonic gonad. Physiol Rev 1986;66:71–117.

(96) Moore KL, Persaud TVN. The developing human: clinically oriented embryology. 6th ed. Philadelphia (PA): Saunders; 1998.

(97) Akhurst RJ, Balmain A. Genetic events and the role of TGF β in epithelial tumour progression. J Pathol 1999;187:82–90.

(98) Reiss M. Transforming growth factor-β and cancer: a love–hate relationship? Oncol Res 1997;9:447–57.

(99) Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor β in human disease. N Engl J Med 2000;342:1350–8.

(100) Pierce DF Jr, Gorska AE, Chytil A, Meise KS, Page DL, Coffey RJ Jr, et al. Mammary tumor suppression by transforming growth factor beta 1 transgene expression. Proc Natl Acad Sci U S A 1995;92:4254–8.

(101) Tang B, Bottiner E, Bagnall K, Anver M, Wakefield L. Enhanced liver and lung tumorigenesis in TGF-β1 heterozygous knock-out mice following treatment with diethylnitrosamine and phenobarbital [abstract]. Proc Am Assoc Cancer Res 1997;38:584.

(102) Brewer MA, Mitchell MF, Bast RC. Prevention of ovarian cancer. In Vivo 1999;13:99–106.

(103) Guzey M, Sattler C, DeLuca HF. Combinational effects of vitamin D and retinoic acid (all *trans* and 9 *cis*) on proliferation, differentiation, and programmed cell death in two small cell lung carcinoma cell lines. Biochem Biophys Res Commun 1998;249:735–44.

(104) Yanagisawa J, Yanagi Y, Masuhiro Y, Suzawa M, Watanabe M, Kashiwagi K, et al. Convergence of transforming growth factor-beta and vitamin D signaling pathways on SMAD transcriptional coactivators. Science 1999;283:1317–21.

NOTES

Supported in part by Department of Defense grant DAMD 17-98-1-8686.

We acknowledge Dr. Mark M. Adams for the generous provision of ovarian tissues from his primate study, supported by Public Health Service grant R01HL46409 from the National Heart, Lung, and Blood Institute, National Institutes of Health, Department of Health and Human Services.

Manuscript received February 21, 2001; revised October 10, 2001; accepted October 17, 2001.

Impact of Progestin and Estrogen Potency in Oral Contraceptives on Ovarian Cancer Risk

Joellen M. Schildkraut, Brian Calingaert, Polly A. Marchbanks, Patricia G. Moorman, Gustavo C. Rodriguez

Background: **Oral contraceptive (OC) use is associated with a reduced risk of developing ovarian cancer, but the mechanism for the risk reduction has not been well defined. In this study, we investigate the relationship between the progestin and estrogen potency in combination OCs and the risk of developing ovarian cancer.** ***Methods:*** **The study included 390 case subjects with epithelial ovarian cancer and 2865 control subjects, between 20 and 54 years of age, identified from the Cancer and Steroid Hormone Study. Logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for the associations between ovarian cancer risk and combination OC formulations while controlling for potential confounders. All statistical tests were two-sided.** ***Results:*** **With users of high-progestin/high-estrogen potency OC as the referent group, users of low-progestin/high-estrogen potency formulations (adjusted OR = 2.1; 95% CI = 1.2 to 3.7) and low-progestin/low-estrogen potency formulations (adjusted OR = 1.6; 95% CI = 0.9 to 3.0) had a higher risk of ovarian cancer than users of high-progestin/high-estrogen potency formulation. Low-progestin potency OC formulations were associated with a statistically significant higher risk than high-progestin potency formulations (adjusted OR = 2.2; 95% CI = 1.3 to 3.9). This association was seen even among users of short duration.** ***Conclusion:*** **The combination OC formulations with high-progestin potency appear to be associated with a greater reduction in ovarian cancer risk than those with low-progestin potency. Mechanisms underlying this reduction may include inhibition of ovulation and/or some direct biologic effects of the progestin.** **[J Natl Cancer Inst 2002;94:32–8]**

Along with parity, oral contraceptive (OC) use has consistently been associated with a decreased risk of ovarian cancer *(1,2)*. Three or more years of OC use reduces the risk of developing epithelial ovarian cancer by 30%–50% *(1,3–5)*. The association increases with the duration of use and appears to be independent of inherent ovarian cancer risk *(1,6,7)*.

The mechanisms underlying this marked reduction have not been well defined. However, it is commonly believed that ovulation, with its associated disruption and subsequent repair of the ovarian epithelium, can lead to the acquisition of genetic damage in ovarian epithelial cells and, in turn, to ovarian cancer in susceptible individuals *(8–10)*. The "incessant ovulation" hypothesis for ovarian cancer is supported by a large volume of epidemiologic evidence linking ovulation with ovarian cancer risk *(1,5,6,8,11–16)* and by the finding that spontaneous ovarian cancers arise frequently in poultry hens, which ovulate daily *(17)*.

Under the incessant ovulation model, reproductive and hormonal factors, such as OC use and pregnancy, have been presumed to alter ovarian cancer risk mainly via their impact on ovulation. Although this hypothesis is attractive, it fails to completely explain the observed differences in the degree of ovarian cancer risk reduction associated with various factors, such as pregnancy, OC use, breast-feeding, and age at menarche, that would be expected simply on the basis of the number of ovulatory cycles that are inhibited *(1,6)*. In addition, pregnancy is associated with a reduced risk of ovarian cancer, even in women who are known to have ovulatory dysfunction and among those for whom the pregnant state has little impact on the number of lifetime ovulatory cycles *(18)*. Some studies *(19,20)* have reported a relationship between increasing risk of epithelial ovarian cancer and increasing time since last birth. These data support the hypothesis that hormonal factors impact ovarian cancer risk through additional biologic mechanisms unrelated to ovulation inhibition *(21)*.

Recently, a 3-year study in primates demonstrated that the progestin component of an OC has a potent apoptotic effect on the ovarian epithelium, providing support for the hypothesis that OCs may lower ovarian cancer risk via induction of cancer-preventive molecular pathways in the ovary *(22)*. Eighty cynomolgus macaques were randomly allocated into one of four

Affiliations of authors: J. M. Schildkraut, B. Calingaert, P. G. Moorman (Department of Community and Family Medicine and the Duke Comprehensive Cancer Center), G. C. Rodriguez (Department of Obstetrics and Gynecology/ Division of Gynecologic Oncology), Duke University Medical Center, Durham, NC; P. A. Marchbanks, Division of Reproduction Health, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Atlanta, GA.

Correspondence to: Joellen M. Schildkraut, Ph.D., Program of Cancer Prevention, Detection and Control Research, Duke University Medical Center, Box 2949, Durham, NC 27710 (e-mail: schil001@mc.duke.edu).

See "Notes" following "References."

groups, including a control group, a group treated with the OC Triphasil (which contains the estrogen ethinyl estradiol and the progestin levonorgestrel), and one group each treated either with ethinyl estradiol or levonorgestrel alone on the same dosage and schedule as those animals receiving Triphasil. At trial completion, examination of the ovaries revealed a striking and statistically significant increase in the percentage of apoptotic ovarian epithelial cells of monkeys treated with Triphasil (14.5%) or levonorgestrel (24.9%) as compared with controls (3.8%) or with monkeys treated with estrogen alone (1.8%). The apoptosis pathway is one of the most important *in vivo* mechanisms for eliminating cells that have sustained DNA damage and are thus prone to malignant transformation *(23)*. In addition, induction of apoptosis is a biologic effect associated with many known chemopreventive agents *(24–31)*. The finding that progestins activate this critical pathway in the ovarian epithelium raises the possibility that progestin-mediated apoptotic effects, and not solely ovulation inhibition as has been previously assumed, may underlie the reduction in ovarian cancer associated with routine OC use. Consistent with these findings, a review and reanalysis of the literature by Risch *(32)* supported the theory that progesterone may render a protective effect on ovarian cancer risk. If this hypothesis is correct, then it is possible that OC formulations that have greater progestin potency may confer greater ovarian cancer protection than OC formulations containing weak progestins.

Only a few case–control studies *(3,5,33,34)* have attempted to examine the relationship between use of specific OC hormonal formulations and ovarian cancer risk. Overall, these studies have shown that combination estrogen–progestin OCs are associated with a reduced risk of ovarian cancer. However, none was able to demonstrate that there was a relationship between hormone potency and this protective effect. Each of these studies has had methodologic limitations, which may have affected their ability to detect meaningful differences in protective efficacy between different OC formulations. An initial analysis of the Cancer and Steroid Hormone (CASH) Study attempted to characterize the protective effect of specific OC formulations on ovarian cancer risk *(5)*. All of the formulations examined appeared to be associated with a reduced risk. However, OC formulations were not categorized according to the potency, or dosages, of estrogen and progestin and there were too few cases of each formulation to detect differences. Similarly, Rosenberg et al. *(3)* suggested a protective effect of progestogen-only contraceptives but did not calculate odds ratios (ORs) for different OC formulations because of the small number of women taking any given formulation. Rosenblatt et al. *(33)* reported a somewhat lower risk reduction associated with low- versus high-potency OC formulations, but the differences were small and could have occurred by chance. In addition, OC formulations were ranked as low versus high potency solely on the basis of the estrogen component, with no consideration of the progestin component. Finally, a recent study by Ness et al. *(34)* suggested that there were no differences in the risk reduction associated with OCs of varying estrogenic and progestin potencies. To our knowledge, this was the largest study, to date, for which hormone potency was taken into account *(34)*.

In this study, we examine the relationship between progestin and estrogen potency and the risk of epithelial ovarian cancer in a reanalysis of the CASH Study data. In this article, consideration is given to the associations with combined estrogen and progestin OCs according to the relative potency of each formulation's subcomponents. Unlike the prior analysis of the CASH Study data, formulations have been categorized and combined according to hormonal potency to have sufficient power to permit the detection of differences in various OC formulations and their association with a reduction in ovarian cancer risk.

Materials and Methods

Study Subjects

Details of the CASH Study have been described previously *(35)*. The ovarian cancer case subjects in this article include patients, 20–54 years of age, diagnosed with epithelial ovarian cancer from December 1, 1980, through December 31, 1982, who participated in the CASH Study. Incident cases of histologically confirmed ovarian cancer were identified from eight population-based tumor registries of the Surveillance, Epidemiology, and End Results (SEER)[1] Program, including the metropolitan areas of Atlanta, GA, Detroit, MI, San Francisco, CA, and Seattle, WA; the states of Connecticut, New Mexico, and Iowa; and four urban counties of Utah. Of the 816 women who were identified as eligible ovarian cancer subjects, 579 (71%) were interviewed. Because of known epidemiologic differences in epithelial versus nonepithelial ovarian cancer, the current analysis was restricted to women classified as having epithelial tumors. At the time of patient accrual for the CASH Study, an expert panel of three pathologists reviewed histologic material from 449 of the epithelial ovarian cancer subjects. Because the classification of tumors as epithelial versus nonepithelial by the panel agreed closely with the original classification by the local pathologists at the time of diagnosis, the classification by the local pathologists was used whenever histologic materials were not available to the panel. Women diagnosed with ovarian cancers of low malignant potential were included in the current study. Previous reports have shown that OC use is associated with a risk reduction for both invasive cancers and tumors of low malignant potential *(36,37)*. Information on the subject's tumor behavior, invasive versus low malignant potential, was available for only the 449 subjects who were reviewed by the three study pathologists. Thus, 324 women with invasive ovarian cancer, 123 with tumors of low malignant potential, two with carcinoma *in situ*, and 44 for whom tumor behavior was unknown were considered for inclusion in the analysis, for a total of 493 women diagnosed with epithelial ovarian cancer available in the CASH Study.

Control subjects were aged 20–54 years, had resided in the same eight geographic locations as the case subjects, and were recruited through random-digit telephone dialing. Of the 5698 women selected as control subjects, 4754 (83%) agreed to participate. The control group was restricted to women who were at risk for a first primary ovarian cancer at the time of the interview. Thus, 711 women were excluded because of a history of bilateral oophorectomy, a prior history of ovarian cancer, or uncertainty regarding prior oophorectomy, leaving 4043 control subjects.

In this study, we limited OC users to women who used combination OC pills (containing both an estrogen and a progestin for 21 days each month). Excluded from the analysis were women who did not know if they had ever used OCs for 3 or more consecutive months (two ovarian cancer case subjects and 10 control subjects), those who had used an unknown type of OC

pill (51 ovarian cancer case subjects and 473 control subjects), those who did not know the dose of an OC that they had used (12 ovarian cancer case subjects and 193 control subjects), and those who used a sequential OC (estrogen-only formulation for the first 14–16 days, followed by combination of estrogen and progestin for 5–6 days) (seven ovarian cancer case subjects and 111 control subjects). Women taking sequential OCs were excluded, since the hormone schedule of these formulations is much different than that of combination OCs. In addition, we excluded women who used progestin-only OCs (one case subject and seven control subjects).

Up to seven OC episodes were recorded among subjects classified as users. Each OC episode was categorized according to progestin and estrogen potency, either low or high, according to the scheme described below. Among those who were classified as OC users, only subjects who had a single OC episode or multiple episodes with all OC episodes being from the same hormone-potency category were retained for analysis purposes. In other words, women who used OCs from more than one potency category were excluded. Altogether, there were 390 epithelial ovarian cancer case subjects and 2865 control subjects available for the analysis.

Data Collection and Analysis Variables

A standardized questionnaire was administered in the home of each study participant. Women who reported three or more consecutive months of OC use were categorized as ever users and women who used OCs for less than 3 months were classified as nonusers. Detailed information on the formulations used was collected from all of the women who reported having used OCs for 3 or more consecutive months. A life calendar (a calendar on which to record major life events around which contraceptive use might be better remembered) and color photographs of OC packages were used to help women recall their contraceptive use up to the time of diagnosis (for case subjects) or the date of the interview (for control subjects).

Additional questionnaire items included socioeconomic information, age at menarche and menopause, use of other hormones, infertility (defined as failure to conceive after 2 years that was determined by a physician to be because of a problem in the woman or both the woman and her partner), number of pregnancies 6 or more months in duration, history of breast-feeding, medical history, and family history of cancer. Reference age was defined as age at diagnosis for women diagnosed with ovarian cancer and age at interview for control subjects.

Strategy for Classifying OC Hormonal Potency

Each OC used by the study participants was classified according to estrogen and progestin potency. Using the categorization described in a standard pharmacy reference text, progestin potency was based on delay of menses and glycogen incorporation in human endometrial vacuoles tests *(38,39)*. For our analyses, OCs classified by the standard text in the low- and intermediate-progestin potency categories were combined into the low-progestin potency category, and the remainder were classified as high potency. For estrogen potency, it was assumed that ethinyl estradiol is twice as potent as mestranol *(40)*. OC formulations containing 35 μg or less of ethinyl estradiol or its equivalent were categorized as low-estrogen potency, and the remainder were classified as high potency. Therefore, each OC formulation was placed in one of four categories: high progestin/high estrogen, high progestin/low estrogen, low progestin/high estrogen, or low progestin/low estrogen (Table 1). The high-potency progestin formulations reported by subjects in Table 1 were first released on the market between 1960 and 1970, with the majority from the period 1966 through 1970 (Demulen; Ovulen; Ovral; Enovid, 10 mg; and Provest) *(41)*. Similarly, among the low-potency progestin formulations, the year of release on the market ranged from the period 1962 through 1975, the majority of which were released from the period 1962 through 1968 (i.e., Enovid, 5 mg; Enovid-E; Norinyl 1 + 80; Ortho-Novum 1/80; Norinyl, 2 mg; Ortho-Novum, 2 mg; Norlestrin, 1 mg; Norlestrin, 2.5 mg; Norinyl 1 + 50; Ovral var brown; and Ovral var blue) *(41)*.

Statistical Analysis

Pearson chi-square tests were used to identify statistical differences between case and control groups for dichotomous variables and nonordinal categorical variables. The extended Mantel–Haenszel chi-square test was used to identify differences between case and control groups for ordinal categoric variables. Student's t tests were used to compare differences between groups for continuous variables. Unconditional logistic regression was used to calculate ORs and 95% confidence intervals (CIs). When assessing the impact of various potency categories of OC formulations relative to nonusers, the potential confounders (reference age, total months or duration of OC use, time since first use or latency of OC use, diabetes, number of pregnancies >6 months, race, infertility, and years of education) were included one at a time in the logistic model and tested to see if they had an impact on the point estimates of the ORs. Those variables causing a 10% change in any of the ORs were included in the final models. In addition, duration of OC use was added to logistic models when comparing various categories of OC formulations with each other. All statistical tests were two-sided.

Results

The frequency distribution of the episodes of use among the various OC formulation categories for the women in the study is shown in Table 1. Although the analysis was limited to those women who used OCs from one potency category, individual subjects included in this analysis used up to five different types or brands within the same potency category of OCs.

No significant differences were found in the reference age or the age at menarche between case and control subjects (Table 2). The mean age at diagnosis for women with ovarian cancer was 43.8 years (standard deviation [SD] = 8.9 years), while the mean age at interview for the control subjects was 44.1 years (SD = 8.2 years). The mean age of menarche was 12.7 years for both the case and control groups, respectively. However, compared with control women, women with epithelial ovarian cancer were more likely to be white, to have 12 or fewer years of education, to have undergone natural menopause versus surgical menopause, and to report having infertility. In addition, the women diagnosed with ovarian cancer had fewer pregnancies and were more likely to report a family history of breast or ovarian cancer in a first-degree relative.

Crude and adjusted ORs for the relationship between ovarian cancer risk and use of OCs according to each potency category as well as any prior use of OCs are presented in Table 3. Using high-progestin/high-estrogen potency OC users as the referent group and controlling for the effect of age, number of pregnan-

Table 1. Oral contraceptive (OC) formulation classifications and frequency distributions of episodes of OC use in the Cancer and Steroid Hormone Study*

OC formulations†	Progestin, mg	Estrogen, μg	Frequency‡	
			No. of case subjects	No. of control subjects
High progestin/high estrogen				
Demulen; Ovulen 50	ED, 1.0	EE, 50	5	50
Ovulen	ED, 1.0	ME, 100	6	163
Enovid, 10 mg	NEL, 10.0	ME, 150	2	8
Ovral	NO, 0.5	EE, 50	10	140
Provest	MPA, 10.0	EE, 50	1	2
High progestin/low estrogen				
Norinyl, 10 mg; Ortho-Novum, 10 mg	N, 10.0	ME, 60	0	17
Low progestin/high estrogen				
ORF 1557-BA	N, 0.5	EE, 50	0	1
Ovcon 50	N, 1.0	EE, 50	0	6
Norinyl 1 + 80; Ortho-Novum 1/80	N, 1.0	ME, 80	17	182
Norinyl, 2 mg; Ortho-Novum, 2 mg	N, 2.0	ME, 100	13	135
Norlestrin, low dose	NA, 0.5	EE, 50	1	4
Norlestrin, 1 mg	NA, 1.0	EE, 50	5	30
Norlestrin, 2.5 mg	NA, 2.5	EE, 50	3	22
Enovid-E	NEL, 2.5	ME, 100	10	179
Enovid, 5 mg	NEL, 5.0	ME, 75	5	50
Low progestin/low estrogen				
Ovcon 35	N, 0.4	EE, 35	0	0
Brevicon; Modicon; ORF 1557-BE	N, 0.5	EE, 35	6	86
Brevicon (1 + 35); Neocon; ORF 1557-BF	N, 1.0	EE, 35	0	1
Norinyl 1 + 50; Noriday; Ortho-Novum 1/50	N, 1.0	ME, 50	23	217
Norlestrin var blue	NA, 0.6	EE, 30	0	1
Loestrin 1/20	NA, 1.0	EE, 20	0	12
Loestrin 1/20; Zorane 1.5/30	NA, 1.5	EE, 30	1	9
Norlestrin var green				
Ovral var brown	NO, 0.2	EE, 15	0	0
Ovral var blue	NO, 0.2	EE, 30	1	3
Lo/Ovral	NO, 0.3	EE, 30	5	52
Total			1280	114

ED = ethynodiol diacetate; EE = ethinyl estradiol; ME = mestranol; NEL = northynodrel; NO = norgestrel; MPA = medroxyprogesterone acetate; N = norethindrone; NA = norethindrone acetate.

†The use of brand names is for identification purposes only and does not imply endoresement by the Centers for Disease Control and Prevention or the U.S. Department of Health and Human Services.

‡Frequency represents the number of OC use episodes of a particular OC. Each OC user had up to five episodes of OC use, with each episode potentially involving a different brand of OC. Thus, the total of 1280 episodes among control subjects and 114 among case subjects is greater than the number of study subjects who used OCs (104 case subjects and 1154 control subjects).

cies, and duration and latency period of OC use, the associations suggested that low-progestin/high-estrogen potency formulations (adjusted OR = 2.1; 95% CI = 1.2 to 3.7) and low-progestin/low-estrogen potency formulations (adjusted OR = 1.6; 95% CI = 0.9 to 3.0) are less protective than high-progestin/high-estrogen potency formulations. Nonusers of OCs were more likely to develop ovarian cancer than high-progestin/high-estrogen potency OC users (OR = 2.9; 95% CI = 1.8 to 4.5) controlling for age and number of pregnancies. In addition, nonusers of OCs were more likely to develop ovarian cancer compared with any potency category of OC users.

Collapsing the data over estrogen potency category and controlling for the effect of estrogen potency, age, the number of pregnancies, and duration and latency period of OC use, low-progestin potency OC formulations were associated with a significantly lower protective effect than high-progestin formulations (adjusted OR = 2.2; 95% CI = 1.3 to 3.9) (Table 3). The relative protective effect of high-potency progestin OCs compared with low-potency progestin OCs remained consistent when the data were stratified according to parity (parous versus nulliparous), menopausal status (premenopausal versus postmenopausal), and tumor behavior (borderline versus malignant) (data not shown). However, the number of subjects in these subcategories who used high-potency progestins was small, producing unstable estimates. Comparison of the relationship between high- and low-estrogen potency formulations and the risk of ovarian cancer, while controlling for progestin potency, age, number of pregnancies, and duration and latency period of OC use, suggested no effect of estrogen potency on ovarian cancer risk, with the risk reduction due to low-potency estrogen formulations similar to that of high-estrogen potency formulations (adjusted OR = 0.7; 95% CI = 0.4 to 1.2) (Table 3).

Further analysis of the association between progestin and estrogen potency and ovarian cancer risk, according to the duration of OC use (3–18 months, 19–59 months, and ≥60 months versus nonusers as the referent), is reported in Table 4. For both high- and low-progestin potency formulations, there was a trend toward an increased protective association with increased duration of use. The results revealed that the protective association of high-potency progestin formulation was greater than low-potency formulations within each category for duration of use, although the CIs overlapped. Among users of high-potency progestin formulations, a statistically significant and markedly reduced risk of ovarian cancer was observed among all categories of duration of use, even among women reporting 3–18 months of OC use (OR = 0.4; 95% CI = 0.2 to 0.8). For the low-potency

Table 2. Comparison of characteristics of 390 case subjects with ovarian cancer and 2865 control subjects participating in the Cancer and Steroid Hormone Study

Characteristic	No. of case subjects (%)	No. of control subjects (%)	P*
Reference age, y			
20–45	184 (47.2)	1328 (46.4)	.759
46–54	206 (52.8)	1537 (53.6)	
Race			
White	348 (89.2)	2366 (82.6)	.001
Nonwhite	42 (10.8)	495 (17.3)	
Missing data		4 (0.1)	
Educational level, y			
≤12	220 (56.4)	1425 (49.7)	.014
>12	170 (43.6)	1437 (50.2)	
Missing data		3 (0.1)	
Pregnancies of 6 mo			
0	100 (25.6)	393 (13.7)	<.001
1	53 (13.6)	273 (9.5)	
2–3	179 (45.9)	1345 (46.9)	
≥4	57 (14.6)	845 (29.5)	
Missing data	1 (0.3)	9 (0.3)	
Age at menarche, y			
≤11	73 (18.7)	609 (21.3)	.350
12–13	217 (55.6)	1521 (53.1)	
>13	97 (24.9)	723 (25.2)	
Missing data	3 (0.8)	12 (0.4)	
Menopausal status			
Premenopausal	155 (39.7)	1160 (40.5)	.002
Perimenopausal	82 (21.0)	614 (21.4)	
Postnatural	91 (23.3)	512 (17.9)	
Postsurgical	41 (10.5)	488 (17.0)	
Missing data	21 (5.4)	91 (3.2)	
Infertility†			
Yes	26 (6.7)	120 (4.2)	.025
No	361 (92.6)	2738 (95.6)	
Missing data	3 (0.8)	7 (0.2)	
History of sugar diabetes			
Yes	15 (3.8)	139 (4.9)	.375
No	375 (96.2)	2719 (94.9)	
Missing data		7 (0.2)	
1st-degree relatives with breast or ovarian cancer			
Yes	46 (11.8)	204 (7.1)	.001
No	344 (88.2)	2661 (92.9)	

*Pearson chi-square test.

†Unsuccessfully tried to get pregnant for 2 years; a physician determined that it was because of a problem in the woman or in both the woman and the partner.

group, a marked reduced risk was apparent only for users for at least 60 months. With regard to estrogen-formulation potency, a similar but weaker trend was observed among high-estrogen potency users compared with high-progestin potency users. There was no consistent relationship among low-estrogen potency users.

Discussion

Our analyses of data from the CASH Study identified an association consistent with a reduced risk of ovarian cancer among users of all formulations of combination OCs, regardless of the hormonal content or potency. When comparing OCs categorized by estrogen and progestin potency, our results provide evidence that OC formulations with higher progestin potency confer a greater reduction in risk of ovarian cancer than those with lower progestin potency, irrespective of the estrogen content, duration of use, and latency. Analyses examining duration of use demonstrate a statistically significant reduction in risk associated with use of high-progestin OCs, even among women who used them for a relatively short duration. Because all of the OC formulations included in the analyses contained both progestin and estrogen, it is not possible to completely separate the effects of the two hormones on ovarian cancer risk. The finding that the degree of protection associated with OC use is related to progestin potency is consistent with the hypothesis that direct biologic effects related to the progestin component may be a mechanism underlying the reduction in ovarian cancer risk associated with OC use.

Since some previously published data support that there is a latency effect of OCs on the decreased risk of ovarian cancer *(3,33)*, one might hypothesize that our results were driven by a greater latency period among high-potency progestin users compared with low-potency progestin users. In fact, we found that, in our data high-potency users had a shorter latent period of OC use (age-adjusted mean = 13.4 years; SD = 3.9 years) than that of low-progestin potency users (age-adjusted mean = 14.3 years; SD = 3.9) ($P<.001$). In addition, controlling for latency did not appear to explain the differences we detected.

It has long been hypothesized that the protective association between OC use and ovarian cancer is related to OC suppression of ovulation, thereby reducing the amount of genetic damage to the ovarian epithelium associated with ovulation. If this hypothesis is correct, all combination estrogen/progestin OCs should be equally protective against ovarian cancer, since they are all potent inhibitors of ovulation. In addition, it might be anticipated that the risk reduction afforded by a short course of OC use would be low, but that it would increase in proportion to the duration of use, as more ovulations are prevented. The results of this study are inconsistent with the hypothesis that OC use is associated with a risk reduction solely through ovulation inhibition, in that we found that the protective association is influenced by the progestin potency of the formulation. Moreover, we found a protective association with use of high-progestin potency OCs, even when used for only a short interval during which few ovulatory cycles are inhibited. The findings supported the conclusions derived from a previous primate study in which the progestin component of OCs was specifically noted to activate chemopreventive molecular pathways in the ovarian epithelium leading the authors to hypothesize that progestins may be effective ovarian cancer preventives *(22)*.

The results of a recent study by Ness et al. *(34)* are not consistent with those of this article. In the former study *(34)*, which included 767 ovarian cancer case subjects and 1367 control subjects, the risk reduction associated with use of low-estrogen/low-progestin pills was identical to that associated with use of high-estrogen/high-progestin pills, with ORs of 0.5 for the risk reduction associated with the use of OCs of each potency class compared with nonuse. Despite similarities between this study and the study by Ness et al. *(34)*, including that both were population-based studies of newly diagnosed ovarian cancer patients and that both used in-person structured interviews, life-events calendars, and pictorial views of OC preparations, there are several aspects of the study design by Ness et al. that suggest why different findings may have arisen. The current study involved younger women, there were temporal differences in the OCs available, and the classification schemes for defining po-

Table 3. Crude and adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for ovarian cancer according to oral contraceptive (OC) hormone potency, with the high-potency group as a referent group in the Cancer and Steroid Hormone Study

Hormone potency	No. of case subjects	No. of control subjects	Crude OR	(95% CI)	Adjusted OR*	(95% CI)
Progestin/estrogen						
High/high	22	334	1.0	(referent)	1.0	(referent)
High/low	0	17	0.0	(0.0 to n/e†)	0.0	(0.0 to n/e†)
Low/high	49	497	1.5	(0.9 to 2.5)	2.1	(1.2 to 3.7)
Low/low	33	306	1.7	(1.0 to 3.0)	1.6	(0.9 to 3.0)
Nonusers	286	1711	2.5	(1.6 to 4.0)	2.9	(1.8 to 4.5)
Progestin						
High	22	351	1.0	(referent)	1.0	(referent)
Low	82	803	1.6	(1.0 to 2.7)	2.2	(1.3 to 3.9)
Nonuser	286	1711	2.7	(1.7 to 4.2)	3.0	(1.9 to 4.7)
Estrogen						
High	71	831	1.0	(referent)	1.0	(referent)
Low	33	323	1.2	(0.8 to 1.8)	0.7	(0.4 to 1.2)
Nonuser	286	1711	2.0	(1.5 to 2.6)	2.0	(1.5 to 2.7)

*ORs for progestin/estrogen potency are adjusted for reference age, number of pregnancies over 6 months, duration in months of OC use, and years since first OC use (latency). ORs for progestin potency are adjusted for all of the above as well as for estrogen level; ORs for estrogen potency are adjusted for all of the above as well as for progestin level. ORs for nonusers are adjusted for reference age and number of pregnancies over 6 months.

†Upper limit of CI not estimable (n/e). Two sided *P* value for high/low versus high/high group from Fisher's exact test was .62.

Table 4. Odds ratios (ORs)* and 95% confidence intervals (CIs) for ovarian cancer according to oral contraceptive (OC) hormone potency, by duration of OC use, with adjustment for age and nonusers group used as a referent in the Cancer and Steroid Hormone Study

	No. of case subjects	No. of control subjects	OR (95% CI)	No. of case subjects	No. of control subjects	OR (95% CI)
Nonusers	286	1711	1.0 (referent)	286	1711	1.0 (referent)
Progestin		High potency			Low potency	
Duration, mo						
3–18	6	88	0.4 (0.2 to 0.8)	30	244	0.7 (0.4 to 1.0)
19–59	8	124	0.3 (0.2 to 0.7)	31	252	0.7 (0.4 to 1.0)
≥60	6	138	0.2 (0.1 to 0.5)	20	296	0.4 (0.2 to 0.6)
Estrogen		High potency			Low potency	
Duration, mo						
3–18	24	214	0.6 (0.4 to 0.9)	12	118	0.5 (0.3 to 1.0)
19–59	24	281	0.5 (0.3 to 0.7)	15	95	0.8 (0.5 to 1.5)
≥60	21	326	0.4 (0.2 to 0.6)	5	108	0.3 (0.1 to 0.6)

*ORs comparing users of high and low potencies of both progestin and estrogen to nonusers.

tency were not identical. It is not clear if these differences could explain the variations in findings between the two studies.

Despite some significant strengths in the design of the CASH Study, limitations of our analysis include the possible misclassification of OC use among ovarian cancer case and control subjects, particularly in terms of the retrospective reporting of specific OC formulations that the study subjects had used in their lifetime. In addition, we did not have formulation and dosage information on all OC users in the CASH Study and did not have an adequate number of women who used progestin-only OCs to examine their effect. The women who participated in the CASH Study were relatively young compared with women in the general population who develop ovarian cancer, and we do not know whether our results apply to menopausal women who develop ovarian cancer. Since the CASH Study was conducted more than 20 years ago, we were unable to evaluate more recent OC formulations. However, the newer formulations have had lower potencies and, therefore, are likely to have a reduced effect on the risk of ovarian cancer.

Despite these limitations, these data provide further support for the hypothesis that biologic effects related to the progestin component in OCs may be a mechanism underlying their protective effect independent of inhibition of ovulation. It is hoped that further research in the field of ovarian cancer prevention will lead to the identification of promising agents, in addition to progestins, which activate cancer-prevention pathways in the ovarian epithelium, and that can then be formulated into a pharmacologic strategy that achieves maximum protection against ovarian cancer, while minimizing side effects.

References

(1) Whittemore AS, Harris R, Intyre J. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case–control studies. II. Invasive epithelial ovarian cancers in white women. Collaborative Ovarian Cancer Group. Am J Epidemiol 1992;136:1184–203.

(2) Weiss NS, Cook LS, Farrow DC, Rosenblatt KA. Ovarian cancer. In: Schottenfeld D, Fraumeni JF Jr, editors. Cancer epidemiology and prevention. New York (NY): Oxford University Press; 1996. p. 1040–57.

(3) Rosenberg L, Palmer JR, Zauber AG, Warshauer ME, Lewis JL, Strom BL, et al. A case–control study of oral contraceptive use and invasive ovarian cancer. Am J Epidemiol 1994;139:654–61.

(4) Weiss NS, Lyon JL, Liff JM, Vollmer WM, Daling JR. Incidence of ovarian cancer in relation to the use of oral contraceptives. Int J Cancer 1981; 28:669–71.

(5) The reduction in risk of ovarian cancer associated with oral-contraceptive use. The Cancer and Steroid Hormone Study of the Centers for Disease Control and the National Institute of Child Health and Human Development. N Engl J Med 1987;316:650–5.

(6) Risch HA, Weiss NS, Lyon JL, Daling JR, Liff JM. Events of reproductive life and the incidence of epithelial ovarian cancer. Am J Epidemiol 1983; 117:128–39.
(7) Narod SA, Risch H, Moslehi R, Dorum A, Neuhausen S, Olsson H, et al. Oral contraceptives and the risk of hereditary ovarian cancer. Hereditary Ovarian Cancer Clinical Study Group. N Engl J Med 1998;339:424–8.
(8) Schildkraut JM, Bastos E, Berchuck A. Relationship between lifetime ovulatory cycles and overexpression of mutant p53 in epithelial ovarian cancer. J Natl Cancer Inst 1997;89:932–8.
(9) Ames BN, Gold LS. Too many rodent carcinogens: mitogenesis increases mutagenesis. Science 1990;249:970–1.
(10) Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE. Increased cell division as a cause of human cancer. Cancer Res 1990;50:7415–21.
(11) Cramer DW, Hutchinson GB, Welch WR, Scully RE, Ryan KJ. Determinants of ovarian cancer risk. I. Reproductive experiences and family history. J Natl Cancer Inst 1983;71:711–6.
(12) Joly DJ, Lilienfeld AM, Diamond EL, Bross ID. An epidemiologic study of the relationship of reproductive experience to cancer of the ovary. Am J Epidemiol 1974;99:190–209.
(13) Hildreth NG, Kelsey JL, LiVolsi VA, Fischer DB, Holford TR, Mostow ED, et al. An epidemiologic study of epithelial carcinoma of the ovary. Am J Epidemiol 1981;114:398–405.
(14) Franceschi S, La Vecchia C, Helmrich SP, Mangioni C, Tognoni G. Risk factors for epithelial ovarian cancer in Italy. Am J Epidemiol 1982;115: 714–9.
(15) Rosenberg L, Shapiro S, Slone D, Kaufman DW, Helmrich SP, Miettinen OS, et al. Epithelial ovarian cancer and combination oral contraceptives. JAMA 1982;247:3210–2.
(16) Whittemore AS, Harris R, Itnyre J, Halpern J. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case–control studies. I. Methods. Collaborative Ovarian Cancer Group. Am J Epidemiol 1992;136: 1175–83.
(17) Fredrickson TN. Ovarian tumors of the hen. Environ Health Perspect 1987; 73:35–51.
(18) Mosgaard BJ, Lidegaard O, Andersen AN. The impact of parity, infertility and treatment with fertility drugs on the risk of ovarian cancer. Acta Obstet Gynecol Scand 1997;76:89–95.
(19) Cooper GS, Schildkraut JM, Whittemore AS, Marchbanks PA. Pregnancy recency and risk of ovarian cancer. Cancer Causes Control 1999;10: 397–402.
(20) Albrektsen G, Heuch I, Kvale G. Reproductive factors and incidence of epithelial ovarian cancer: a Norwegian prospective study. Cancer Causes Control 1996;7:421–7.
(21) Adami HO, Hsieh CC, Lambe M, Trichopoulos D, Leon D, Persson I, et al. Parity, age at first childbirth, and risk of ovarian cancer. Lancet 1994;344: 1250–4.
(22) Rodriguez GC, Walmer DK, Cline M, Krigman H, Lessey BA, Whitaker RS, et al. Effect of progestin on the ovarian epithelium of macaques: cancer prevention through apoptosis? J Soc Gynecol Investig 1998;5:271–6.
(23) Canman CE, Chen CY, Lee MH, Kastan MB. DNA damage responses: p53 induction, cell cycle perturbations, and apoptosis. Cold Spring Harb Symp Quant Biol 1994;59:277–86.
(24) Ponzoni M, Bocca P, Chiesa V, Decensi A, Pistoia V, Raffaghello L, et al. Differential effects of *N*-(4-hydroxyphenyl)retinamide and retinoic acid on neuroblastoma cells: apoptosis versus differentiation. Cancer Res 1995;55: 853–61.
(25) Delia D, Aiello A, Lombardi L, Pelicci PG, Grignani F, Formelli F, et al. *N*-(4-Hydroxyphenyl)retinamide induces apoptosis of malignant hemopoietic cell lines including those unresponsive to retinoic acid. Cancer Res 1993;53:6036–41.
(26) Lotan R. Retinoids in cancer chemoprevention. FASEB J 1996;10:1031–9.
(27) Kuo SM. Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells. Cancer Lett 1996;110:41–8.
(28) Thompson HJ, Jiang C, Lu J, Mehta RG, Piazza GA, Paranka NS, et al. Sulfone metabolite of sulindac inhibits mammary carcinogenesis. Cancer Res 1997;57:267–71.
(29) Gould MN. Cancer chemoprevention and therapy by monoterpenes. Environ Health Perspect 1997;105 Suppl 4:977–9.
(30) Pascale RM, Simile MM, De Miglio MR, Nufris A, Daino L, Seddaiu MA, et al. Chemoprevention by *S*-adenosyl-L-methionine of rat liver carcinogenesis initiated by 1,2-dimethylhydrazine and promoted by orotic acid. Carcinogenesis 1995;16:427–30.
(31) el-Bayoumy K, Upadhyaya P, Chae YH, Sohn OS, Rao CV, Fiala E, et al. Chemoprevention of cancer by organoselenium compounds. J Cell Biochem Suppl 1995;22:92–100.
(32) Risch HA. Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. J Natl Cancer Inst 1998;90:1774–86.
(33) Rosenblatt KA, Thomas DB, Noonan EA. High-dose and low-dose combined oral contraceptives: protection against epithelial ovarian cancer and the length of the protective effect. The WHO Collaborative Study of Neoplasia and Steroid Contraceptives. Eur J Cancer 1992;28A:1872–6.
(34) Ness RB, Grisso JA, Klapper J, Schlesselman JJ, Silberzweig S, Vergona R, et al. Risk of ovarian cancer in relation to estrogen and progestin dose and use characteristics of oral contraceptives. SHARE Study Group. Steroid Hormones and Reproductions. Am J Epidemiol 2000;152:233–41.
(35) Wingo PA, Ory HW, Layde PM, Lee NC. The evaluation of the data collection process for a multicenter, population-based, case–control design. Am J Epidemiol 1988;128:206–17.
(36) Risch HA, Marrett LD, Jain M, Howe GR. Differences in risk factors for epithelial ovarian cancer by histologic type. Am J Epidemiol 1996;144: 363–72.
(37) Parazzini F, Restelli C, La Vecchia C, Negri E, Chiari S, Maggi R, et al. Risk factors for epithelial ovarian tumours of borderline malignancy. Int J Epidemiol 1991;20:871–7.
(38) Covington TR, Dipalma JR, Hussar DA, Lasagna L, Tatro DS, Whitsett TL, editors. Hormones. In: Drug facts and comparisons. St. Louis (MO): J. B. Lippincott Co.; 1986.
(39) Piper JM, Kennedy DL. Oral contraceptives in the United States: trends in content and potency. Int J Epidemiol 1987;16:215–21.
(40) Bolt HM, Bolt WH. Pharmacokinetics of mestranol in man in relation to its oestrogenic activity. Eur J Clin Pharmacol 1974;7:295–305.
(41) Hatcher RA, Stewart GK, Guest F, Finkelstein R, Godwin C. Contraceptive technology, 1976–1977. 8th ed. New York (NY): Irvington; 1976. p. 42–3.

NOTES

[1]*Editor's note:* SEER is a set of geographically defined, population-based, central cancer registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Registry data are submitted electronically without personal identifiers to the NCI on a biannual basis, and the NCI makes the data available to the public for scientific research.

Supported by Public Health Service grant CA76016 from the National Cancer Institute (NCI), National Institutes of Health (NIH), Department of Health and Human Services (DHHS), and by grant DAMD 17-98-1-8656 from the Department of Defense. The Cancer and Steroid Hormone Study was supported by interagency agreement 3-01 HD-8-1037 between the Centers for Disease Control and Prevention and the National Institute of Child Health and Human Development, NIH, DHHS, with additional support from the NCI.

We acknowledge the contributions of Drs. Kathryn Curtis (Centers for Disease Control and Prevention, Atlanta, GA) and Andrew Berchuck (Duke University Medical Center, Durham, NC).

Manuscript received January 29, 2001; revised October 3, 2001; accepted October 15, 2001.